BIODIVERSITY STUDY OF HALOPHILES FROM LITTLE RANN OF KUTCH WITH SPECIAL EMPHASIS ON LIPASE PRODUCTION

Ph. D. THESIS

IN SUBJECT OF MICROBIOLOGY

Submitted to:

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Academic year: 2011-12

Submitted by:
Guided by:

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A THESIS

Submitted to:

SAURASHTRA UNIVERSITY, RAJKOT

For the Degree of

DOCTOR OF PHYLOSOPHY

In

THE FACULTY OF SCIENCE (MICROBIOLOGY)

Submitted by:

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The work included in the thesis is my own work under the supervision of Dr. Neepa Pandhi and leads to some contribution in Microbiology subsidized by a number of references.

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Place: Rajkot **(Mrugesh D. Khunt)**

This is to certify that the present work submitted for the Ph.D degree of Saurashtra University by Mrugesh D. Khunt is his own work and leads to advancement in the knowledge of Microbiology. The thesis has been prepared under my supervision.

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Achievement of goal is not one person's job. It requires the efforts of many people together.

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CHAPTER-1

Introduction

The domain Archaea was not discovered as a major domain of life earlier but with the collaborative efforts of Carl Woese and his colleagues, the domain was differentiated from Bacteria. Domain Archaea contain organisms known as extremophiles like thermophiles, acidophiles, halophiles, methanogens, alkalophiles etc. Carl Woese proposed three domains of life on the basis of molecular chronometer 16s r-RNA and 18s r-RNA. They are Bacteria, Archaea and Eukarya. The word archaea or archaeabacteria comes from Greek word meaning "ancient things". Archaea are believed to be most primitive organisms. They are biochemically more closely related to the Eukarya than to the Bacteria (Bullock, 2000).

Extremophiles, the organisms that can grow in extreme environments, have attracted considerable attention in recent years. Life in extreme environments has been studied intensively focusing attention on the diversity of organisms and molecular and regulatory mechanisms involved. Currently, emphasis is given on their population dynamics and molecular phylogeny along with their biotechnological novelty. Members of these groups of organisms may hold secret for origin of life and answer many basic questions about the stability of the macromolecules under extreme conditions. Extremophiles harbor in extreme conditions such as high temperature, extreme pH, high salt concentration etc.

As halophilic means "salt loving", halophilic enzymes or bacteria by definition require high NaCl for activity or growth. In contrast, halotolerant microorganisms require normal NaCl concentration (0.5-1%, w/v) but they can tolerate NaCl concentration up to 5%, w/v (Garabito *et al.*, 1998). Halophiles are able to tolerate high salt concentration by accumulating KCl equal to the external concentration of NaCl. They are normally found in natural habitats, from freshwater environments to hypersaline lakes such as the Dead Sea, Salt crystallizer ponds, other places saturated with sodium chloride, salt deserts etc. Several halophilic Archaea have been isolated from various habitats, for example, *Halobaculum gomorrense* from Dead Sea (Oren *et al.*, 1995); *Halorhabdus utahensis* from Great Salt Lake (Wanio *et al.*, 2000); and

Halogeometricum borinquense from solar salterns of Puerto Rico (Montalvo-Rodriguez *et al*., 1998). Many strains of *Halobacterium salinarum* have been isolated from habitats like Thai fish sauce, salted fish and hides, solar salterns, estuaries polluted with crude oil etc. (Thongthai *et al*., 1992; Raghavan *et al*., 2000).

Halophiles contains a very large and heterogeneous group of extremophiles (Ventosa *et al*., 1998; Oren, 2002) and have been distinguished on the basis of their salt requirements for growth as extreme halophiles (15–30%, w/v) and moderate halophiles (3–15%, w/v) (Ventosa *et al*., 1998). These groups of the organisms have not been widely explored and hence they have attracted major attention of the scientific community. This is also because of physiological adaptation to highly saline environment and their ecology. Halophilic Bacteria are diversified in terms of physiology i.e. aerobic, anaerobic, chemoheterotrophs, photoautotrophs and photoheterotrophs, as well as chemolithotrophs (Ollivier *et al*., 1994; Oren, 1999). They commonly inhabit the marine environment, esturine, and the salt deserts.

Little *Rann* of Kutch (Kutch desert) is believed to have been a shallow sea and comparatively unexplored field from microbial diversity point of view. The *Rann* of Kutch is an area of 18,000 square km situated within state of Gujarat in India along the border with Pakistan. The Little *Rann* of Kutch extends northeast from the Gulf of Kutch over 5,100 square km. Once an extension of the Arabian Sea, the *Rann* has been closed off by centuries of silting. It is a typical ecosystem with saline desert climate having unique floral and faunal diversity. Little *Rann* of Kutch is believed to have formed due to variety of geomorphic facets of Kutch such as the present surface configuration, its landforms, drainage characteristics and relief pattern. This clearly reveals a complex interplay of tectonics, sea-level changes and lithology as also erosion and deposition.

The Indian Wild Ass Sanctuary is located in the Little *Rann* of Kutch and covers an area of 4954 square km. The Sanctuary is named after a sub species of wild ass (*Equus hemionus khur*), the last population of which it harbours. Wild ass contains halophilic microorganisms as normal flora of their intestine. The grass growing in this region has high salt content due to presence of salt in the land. As wild asses are herbivore, their normal microbial floras have adapted to high salt concentrations and

some might have been replaced due to such salty grass. This indicates that intestinal floras are halophilic and the present work is also focused on study of diversity in intestinal microbial flora due to external conditions.

Hypersaline environments are characterized by high or very high but variable osmotic strength and moderate halophiles or extreme halophiles present in these environments must be able to adapt to the changes in osmotic pressure due to presence of salt. Most halophilic and halotolerant bacteria maintain viability in these environments by accumulating low-molecular weight, water soluble organic compounds known as compatible solutes to counteract the dangerous effect of high salt on cell metabolism and water loss from cell due to difference in osmotic pressure (Louis and Galinski, 1997; Cánovas *et al*., 1998; Bursy *et al*., 2008). Gram-positive and Gram-negative bacteria are known to accumulate compatible solutes like ectoines, glycine and betaine as an osmolytes to counteract action of high salinity (Louis and Galinski, 1997). Osmolytes are synthesized de novo or may be taken up from the external environment and they can be amassed by the cell in very high concentrations to provide osmotic balance without affecting essential cellular functions (Vargas *et al*., 2008; Bursy *et al*., 2008). Osmolytes like ectoines may serve as general stress protectants as they are produced both in responses to salt and heat stresses (Vargas *et al*., 2008; Bursy *et al*., 2008).

Halophilic Bacteria possess a number of interesting applications as well (Ramos-Cormenzana, 1989; Ventosa *et al*., 1998). Many heterotrophic halophilic bacteria can use a wide range of compounds as sole carbon and energy source (Kushner and Kamekura, 1988). In recent era genetic manipulation in halophiles are becoming increasingly available. Many of the halophiles produce industrially valuable compounds such as osmoregulants, enzymes, polymers etc. Most of the halophiles produce extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pullulanases and xylanases which have quite diverse potential usage in different areas such as food industry, feed additive, biomedical sciences and chemical industries (Rao *et al*., 1998; Kulkarni *et al*., 1999; Niehaus *et al*., 1999; Pandey *et al*., 1999). Because of the extreme nature of enzymes they can execute the current requirement of industry.

The use of enzyme as a bioprocess tool can be traced to ancient civilizations. Now a day, nearly 4000 enzymes are known and about 200 are in commercial use. The

majority of commercially used enzymes are of microbial origin. Until the mid 19th century, total enzyme sell was in few million dollars but after then the market grew spectacularly (Wilke, 1999), because of increased understanding of biochemistry, advances in fermentation and downstream process as well as recent advances in genetics and molecular biology. 75% of world supply of enzymes is supplied by Europe only. Protease is dominant in enzyme market and accounts for around 40% of total enzyme sell.

Lipases (Triacylglycerol acylhydrolases E.C.3.1.13) are found in animals, plants and microorganisms (Kamimura *et al.*, 2001; Burkert *et al.*, 2004). Lipase catalyzes the hydrolysis of ester bonds of triacylglycerols to glycerol and free fatty acids at oil-water interface and does not hydrolyze dissolved substrates in the bulk fluid (Sharma *et al.*, 2001). The free fatty acids, especially volatile fatty acids (VFA) were shown to be associated with the aroma and flavor in food products; therefore, lipase has been used for development of flavor and aroma in cheese ripening, bakery products, sausages, yoghurt and beverages (Jaeger *et al.*, 1994; Sharma *et al.*, 2001). Since industrial applications of lipase require specific properties, there is still an interest in additional lipase that could be used in new applications (*Jaeger et al., 1994;* Lambit and Goswami, 2002; Kyu *et al.*, 2005).

Because of their wide scale applications, lipases remain a subject of intensive study (Alberghina *et al.*, 1991; Bornscheuer, 2000). Lipases research is focused particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes and general characterization of performance (Alberghina *et al.*, 1991; Bornscheuer, 2000).

Till date, commercially useful lipases have been obtained from microorganisms that produce a wide variety of extracellular lipases. Many types of lipases are active in organic solvents and catalyze a number of reactions like esterification (Chowdary *et al.*, 2001; Hamsaveni *et al.*, 2001; Kiran *et al.*, 2001; Kiyota *et al.*, 2001; Krishna and Karanth, 2001; Krishna *et al.*, 2001; Rao and Divakar, 2001), transesterification, regioselective acylation of glycols and menthols, synthesis of peptides (Ducret *et al.*, 1998; Zhang *et al.*, 2001) and other chemicals (Therisod and Klibanov, 1987; Weber *et al.*, 1999; Bornscheuer, 2000; Berglund and Hutt, 2000; Liese *et al.*, 2000; Azim *et*

al., 2001). The expectation is that lipases will be as important industrially in the future as the proteases and carbohydrases are currently.

Objectives:

The present work was initiated with the following objectives:

- To collect water, mud and soil samples from various locations in little *Rann* of Kutch and excreta of wild ass from " Indian Wild Ass Sanctuary" located in the Little *Rann* of Kutch
- Enrichment and isolation of Halophiles from the collected samples.
- Morphologically and physiological characterization of Halophiles.
- Screening of lipase, amylase, protease, cellulose and chitinase producing halophiles from the samples.
- Selection of the most potent producer of lipase and optimization of various growth conditions and medium constituents for maximum enzyme production.
- Partial purification of enzyme by ammonium sulfate precipitation.
- Characterization of enzyme with respect to its pH optima, temperature optima, temperature stability, urea denaturation, effect of inorganic salts, Km and Vmax .
- UV mutagenesis for strain improvement.

CHAPTER-2

Review of Literature

Earth is considered to be harboring bewildering diversity of microorganisms. It contains variety of microorganisms with different morphological, physiological and genetical characteristics. Metagenomic studies indicate that only 0.1-1% organisms are cultivable on media and rests of the organisms are uncultured. Current emphasis is on the cultivation of extremophillic organisms in order to explore the unexplored organisms in extreme environment. It provides idea of many unanswered questions related to biology, biochemistry and genetics of organisms that survive in harsh or extreme conditions.

The Archaea constitute one of the three domains of life (Woese *et al.*, 1990), other than Eukaryota and Bacteria, although their origin is subject of debate (Gupta, 2000; Cavalier-Smith, 2002; Gribaldo *et al.*, 2006; Lake *et al.*, 2007). They were earlier believed to inhabit only extreme environments such as extremely hot, or hot and acidic, extremely saline, or very acidic or alkaline conditions (Woese, 1987), but recent studies indicate that they are widespread in different environments (Schleper, 2005). The archaea also include methanogens, that grows under strictly anaerobic and many a times thermophilic conditions, and derive all of their metabolic energy by reduction of organic molecule via methanogenesis. The archaeal species branch distinctly from all other organisms in phylogenetic trees based on 16S rRNA and many other gene and protein sequences (Iwabe *et al.*, 1989; Olsen *et al.*, 1994; Brown and Doolittle, 1997). Most of the Archaea are highly adapted to survive in extreme chemical and physical environments like temperature, pH, salt etc. and the group can be divided into hyperthermophiles, halophiles and methanogens. Despite outward appearances however, the Archaea are more closely biochemically related to the Eukarya than to the Eubacteria (Bullock, 2000).

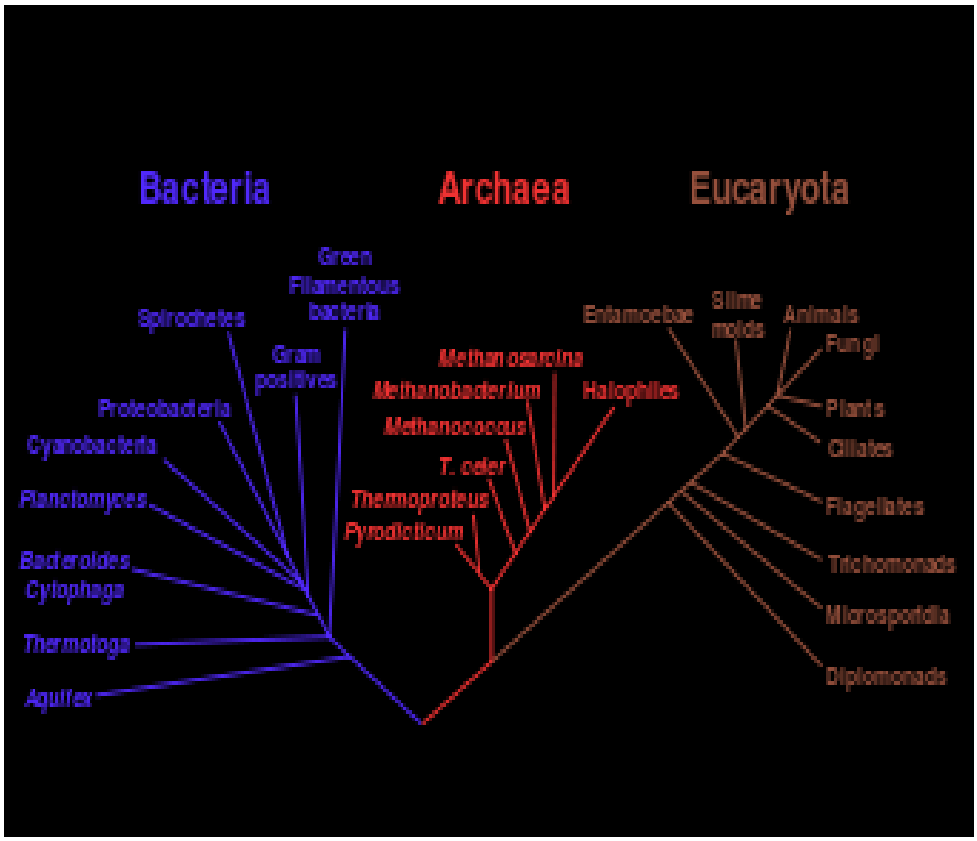


Figure 2.1 Universal Phylogenetic tree

2.1 Phylogeny of an archaea

The domain Archaea contains three major branches: Crenarchaeota (3 orders, 24 genera), Euryarchaeota (10 orders, >50 genera) and Korarchaeota- discovered only on the basis of PCR amplification of 16s r-RNA form natural samples (Woese *et al.*, 1990; Barns *et al.*, 1996). A fourth phylum Nanoarchaeota has been recently discovered (Huber *et al.*, 2002). Following section briefly describes above four phyla.

2.1.1 Crenarchaeota

The word Crenarchaeota is derived from Greek word meaning "spring old quality". Crenarchaeota is also known as Crenarchaea. It has been classified as either a phylum of the Archaea kingdom (Gurtler, 2001; Cavalier-Smith, 2002; Stackebrandt; 2002) or a kingdom of its own (Dalevi *et al.*, 2001). All the cultured Crenarchaea had been thermophilic or hyperthermophilic, isolated from geothermally heated soils or wastes containing elemental sulphur and sulphides; some of which have the ability to grow at up to 113 °C (Blochl *et al.*, 1997). A Crenarchaeota phylum was discovered on the basis of difference in 16S r-RNA sequence from other phyla and lack of histone,

although some Crenarchaeota have histones (Cubonova *et al.*, 2005). Organisms belonging to Crenarchaeota are diverse in shape i.e. rod, cocci, filamentous, irregular etc. and are Gram negative (Garrity and Boone, 2001).

2.1.2 Euryarchaeota

The word Euryarchaeota comes from Greek word meaning "broad old quality" (Michael Hogan, 2010). Phylum Euryarchaeota include methanogens, extreme halophiles, extremely thermophillic aerobes and anaerobes etc. This phylum was separated from other archaea on the basis of 16s rRNA sequence. Methanogens inhabit intestinal tract of animals and sewage treatment plants. Thermophiles and hyperthermophiles inhabit geothermal vent and other high temperature habitats. Halophiles inhabit natural or artificial saline habitats.

2.1.3 Korarcheota

This phylum includes hyperthermophillic organisms. It branches close to root of archaea and hence contains many features of ancient organisms. Until now, little is known about this phylum.

2.1.4 Nanoarcheota

Very recently, from sample of a marine hydrothermal system near Iceland, a co-culture of a new Ignicoccus strain and small archaeal cocci was obtained. The small cocci turned out to represent the first member of a novel archaeal phylum, the "Nanoarchaeota" (Huber *et al.*, 2002). The name of archaea phyla was derived from Greek word meaning "old dwarf" (NCBI web page on Nanoarcheota). This phylum currently has only one representative, *Nanoarchaeum equitans*. The organism is hyperthermophiles and prefers to grow at boiling temperature.

2.2 Classification of halophiles

Phylogenetically halophiles can be placed in following hierarchy,

Domain: Archaea,
Class: Halobacteria,
Order: Halobacteriales
Family: Halobacteriaceae.

There are total 14 valid genera according to Bergey's Manual of Systematic Bacteriology (2001), includes *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natrialba*, *Halobaculum*, *Halogeometricum*, *Haloterrigena*, *Natronorubrum*, *Halorubrum*, *Natronomonas*, *Naronobacterium*, *Natronococcus* and *Natrinema*.

Many criteria may be applied for taxonomic classification of halophiles. One taxonomic criterion for the identification and recognition of haloarchaea is the sequence of the 16S r-RNA genes, specifically signature sequences (Kamekura *et al*. 2004). This is the main criteria for taxonomic classification. Another criterion is the composition of membrane lipids (polar lipids) which can be used as one of the criteria for the classification of different haloarchaeal genera (Ross *et al*. 1985). Data based on both of the above mentioned criteria are consistent with one another (Grant *et al*. 2001). Third way of classification is on the basis of phenotypic characteristics according to Bergey's Manual of Systematic Bacteriology.

2.3 Extremophiles

Extremophiles have been categorized in to various groups on the basis of their survival under different extremities. They grow optimally in some of earth's most hostile environments of temperature (-2ºC to 15ºC; Psychrophiles; and 60ºC to 115ºC; Thermophiles), salinity (2-5M NaCl; Halophiles), pH (<4 Acidophiles and >9; Alkaliphiles), anaerobicity (Methanogens), and/or pressure (Barophiles). Halophiles are aerobic microorganisms that live and grow in high saline/salty environments. The saline content in halophilic environment is usually 10 times the saline/salt content of normal ocean water.

2.4 Halophiles and its environment

Majority of halophiles can inhabit very extreme saline environments such as salt lakes and salt evaporation ponds. Example of such extreme saline environments can be found in the two largest hypersaline lakes; the Great Salt Lake and the Dead Sea. Hypersaline environments are generally defined as those containing salt concentrations in excess of sea water (3.5% total dissolved salts). Hypersaline environments can be divided into two broad categories. These are the thalassohaline and athalassohaline. Thalassohaline water bodies have similar salt composition to seawater with sodium and chloride being the dominant ions; common examples

include the Great Salt Lake in Utah, brine springs from underground salt deposits and solar salterns (Litchfield and Gillevet, 2002). While athalassohaline water bodies such as the Dead Sea, Lake Magadi in Kenya, Wadi Natrun in Egypt, the soda lakes of Antarctica and Big Soda Lake and Mono Lake in California are dominated by potassium, magnesium, or sodium (Oren, 2002). Some of the hypersaline environments are artificially constructed.

Microorganisms living in such saline environments are termed as halophiles and can be divided into **slight halophiles** growing optimally at 0.2-0.85mol/L (2-5%) NaCl e.g. *Methanosalsum zhilinae* (Boone and Baker, 2001), **moderate halophiles** growing optimally at 0.85-3.4 mol/L (5-20%) NaCl, e.g. *Halomonas almeriensis* (Martínez-Checa *et al.*, 2005) and **Extreme halophiles** growing optimally above 3.4-5.1 mol/L (20-30%) NaCl e.g. *Halogeometricum borinquense* (Montalvo-Rodríguez *et al.*, 1998). Many halotolerant microorganisms can grow in a wide range of salt e.g. *Halobacillus yeomjeoni* can grow from 0.5% to 21 %(w/v) NaCl (Yoon *et al.*, 2005).

Table 2.1 Classification of Microorganisms based on salt requirement

Sr. No.	Groups	Types	Growth on media + Salt
1	Non-halophiles	Salt sensitive	Grow in media containing < 2% (W/V) Salt
		Salt tolerance	Grow best in media containing < 2% salt but can able to grow in media containing > 2% salt
2	Halophiles	Facultative	Grow in media containing < 2% salt but grow best in media containing > 2% salt
		Obligate	Grow only in media containing >2% salt.

2.5 Adaptation of halophiles in saline environment

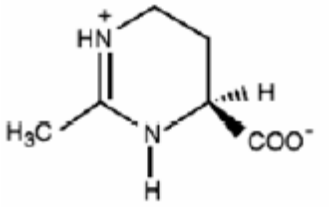
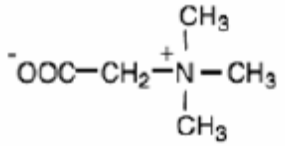
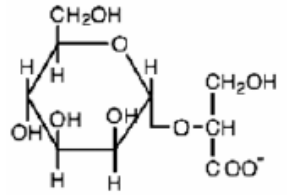
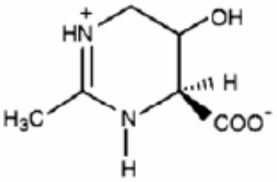
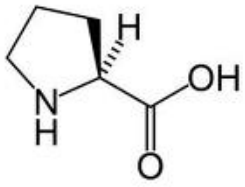
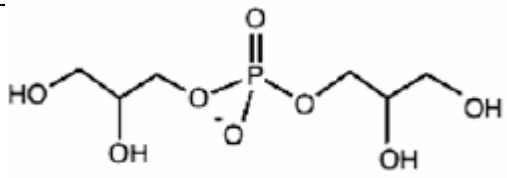
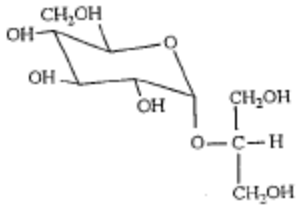
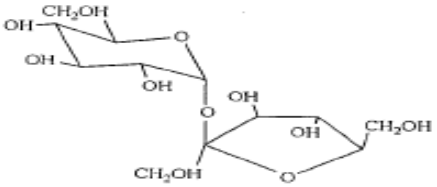
As the plasma membrane of microbes is permeable to water, none of microorganisms living at high salt concentrations can maintain hypo-osmotic intracellular concentration to the osmotic pressure of outer environment. This is applicable to

halophilic microorganisms also. Halophillic microorganisms have been adapted to hypersaline environment by certain mechanisms of maintaining turgor pressure.

For the maintenance of turgor pressure, sodium ions present in the cytoplasm must be excreted outside the cell as much as possible, although mechanisms of sodium poisoning in cells are unclear. Considerable diversity exists among extreme halophiles and moderate halophiles in terms of osmotic adaptation. For the excretion of sodium ion outside, halophiles have Na^+/H^+ antiporter system that expel sodium ion from the interior of the cell (Oren, 1999). Different strategies are used by different groups of halophiles, fundamental of all the strategies remain same i.e. excretion of sodium ion exterior of the cell. Alternate strategy involves accumulation of K^+ and Cl^- ion to maintain osmotic balance. Even Halobacterials, the aerobic halophiles, can accumulate KCl at the same concentration of NaCl in surrounding media. Halophiles have developed alternate strategies for the functioning of enzymes and other biomolecules at such higher salt concentrations in cytoplasm. These adaptation strategies are referred as "Salt-in" strategies.

Another strategy to maintain turgor pressure in hypersaline environment is by accumulating compatible solutes. Compatible solutes are low molecular weight solutes accumulated in cell in high concentration, which acts as osmoprotectant. Sugars, alcohols, amino acids, betaines, ectoines or their derivatives are the common compatible solutes synthesized by halophiles (Ventosa *et al*., 1998). They stabilize enzymes, DNA and whole cell against stresses such as freezing, drying and heating. The concentrations of compatible solutes are regulated according to the salt concentration in which the cells live (Galinski and Louis, 1999). It can be changed according to outside salt concentration. The compounds can also be degraded, transformed or excreted into medium as salt concentration decreases (Trüper and Galinski, 1990). Eukaryotic algae *Dunaliella* accumulate glycerol as compatible solute (Ben-Amotz and Avron, 1973). Glycine and betaine was found to be accumulated by *Halorhodospira halochloris* (Galinski and Trüper, 1982). Apart from all the above described compatible solutes, number of compatible solutes synthesized by halophilic microorganisms steadily increases day by day (Imhoff, 1986; Reed, 1986; Wohlfarth *et al*., 1990).

Table 2.2 Common compatible solutes, producer organisms and its molecular structure

Compatible solute	Produced by	Structure
Ectoine	*Chromohalobacter israelensis, Chromohalobacter salexigens, Halomonas elongata, Halomonas boliviensis, Brevibacterium epidermis, Ectothiorhodospira halochloris.*	
Betaine	*Thioalkalivibrio versutus, Halorhodopira halochloris.*	
Mannosylglycerate	*Thermus thermophilus, Pyrococcus furiosus, Pyrococcus furiosus, Rhodothermus marinus, Methanothermus fervidus.*	
Hydroxyectoine	*Nocardiopsis halophila, Halomonas elongate.*	
Proline	*Bacillus spp.*	
Diglycerol phosphate	*Archaeoglobus fulgidus*	
Glucosylglycerol	*Stenotrophomonas maltophilia, Erwinia chrysanthemi.*	
Sucrose	*Nitrosomonas europaea,* proteobacteria	

2.6 Salient features of halophiles

Halophiles contain certain unique features over non-halophilic organisms. These are:

2.6.1 Cell wall

The major difference between eubacterial call wall and archaebacterial cell wall is that eubacterial cell wall is composed of peptidoglycan (also known as Murein) and archaebacterial cell wall is composed of Pseudopeptidoglycan (also known as Pseudomurein). Cell-wall determines shape of archaea e.g. *Haloferax* in pleomorphic shape, *Halobacterium* in rod shape, *Halococcus, Natronococcus* in spherical shape etc. (Englert *et al.*, 1992). The unusual shapes of archaea are due to the fact that they do not produce significant turgor pressure and hence they contain unique shapes that are not possessed by other groups (Walsby, 1971). *Halococcus* and *Natronococcus* have thick and rigid cell wall that provides structural stability. *Halobacteriaceae* have rigid cell wall containing large glycoprotein subunits referred as S-layer. Glycoprotein is required for cell wall stability against high concentration of NaCl. *Halobacterium* does not contain DAP or teichoic acid found in normal cell (Kushner and Onishi, 1968). Additionally, cell wall of *Halobacterium* contains negatively charged proteins which are stabilized by attracting sodium ions (Na^+) from saline water.

2.6.2 Lipid and cytoplasmic membrane

A cytoplasmic membrane of organism performs several functions like participation in electron transport, movement of ions across membrane, sensory function with environment etc. Biological membrane is composed of proteins, lipids and carbohydrates. The major difference between eubacterial and archaebacterial lipid in cell membrane is that eubacteria contain ester linkage between fatty acids and glycerol while archaebacteria have ether linkage between fatty acids and glycerol backbone. Replacement of ester linkage with ether linkage in archaea provides stability against high salt concentrations. Cell membrane containing ether linkage in lipid was found to be stable at wide temperature range and high salt concentration (Edward, 1990). *Halobacteria* contains archaea specific lipids in cell membrane, composed of 20 carbons (Phytanyl) or 25 carbons (Sestertepanyl), and usually bound to glycerol back bone with ester bond. *Halobacteria* contains neutral or polar lipids in cell membrane. The major phospholipids present in halobacteriaceae are Phosphatidyl

choline, phosphatidyl ethanolamine, cardiolipin, and glycolilids. Amount of phosphatidyl choline and cardiolipin (negatively charged) increases and neutral phospholipids decreases as salt concentration increases (Russell, 1993, Vreeland, 1987). *Salinovibrio costicola* contains 48% phosphatidyl glycerol, 42% phosphatidyl ethanolamine and 10% Cardiolipin (Russell, 1993).

2.6.3 Capsules

Many halophilic bacteria produce extracellular polysaccharide known as capsule. Capsule serves many functions and protect against fluctuation in pH, temperature and other environmental factors. Extracellular polysaccharide of halophiles is viscous at acidic pH. A known halophile, *Halomonas maura* produces extracellular polysaccharide containing carbohydrates and protein (Bouchotroch *et al.*, 2001).

2.6.4 Metabolism in halophiles

Halophiles differ in nutritional requirements. Some of the halophiles require single carbon and nitrogen source while some are fastidious and require complex nutrition including high concentration of yeast extract and other rich organic nutrients. Halophiles are able to ferment large number of sugars with gas production, utilize intermediate compounds and can utilize variety of proteinic sources (Flannery, *et al.*, 1952). Generally, high sodium chloride is found to be lethal for microorganisms because it causes dehydration, removes oxygen, interferes with enzyme, sensitizes carbon dioxide and causes direct action on the cell (Rockwell and Ebertz, 1924). Halophiles have developed many strategies to tolerate lethal action of sodium chloride described in previous section. Certain pigmented halophiles have less ability for substrate utilization and requires more time for growth.

2.7 Biotechnological applications of halophiles

Halophilic microorganisms find number of applications in biotechnology. Although produced by non-halophilic organisms, halophilic products have distinct advantages. Halophilic media are less prone to contamination and produces product with unique properties.

Table 2.3 Biotechnological applications of halophiles

Sr. No.	Product	Use in biotechnology	Producer organism(s)	References
1	Bacteriorhodopsin	Preparation of computer storage memories and processing unit, photoelectric convertors, halographic storage materials, radiation detector, preparation of biosensor	*Halobacterium salinarium*	Margesin and Schinner, 2001
3	Biofuel	As an alternative of fossil fuel	*Dunaliella*	Goldman *et al.*, 1980
4	Compatible solutes	moisturizers in cosmetics, stabilizers in the polymerase chain reaction, Enzyme stabilizer	*Halomonas elongate, Marinococcus.*	Sauer and Galinski, 1998; Motitschke *et al.*, 2000
5	Biosurfactant	Bioremediation of oil polluted site, MEOR,	*Rhodococci, B. axarquiensis B. malacitensis*	Yakimov *et al.*, 1999, Ruiz-García *et al.*, 2005
6	β-carotene	Antioxident, coloring agent, multivitamin preparation	*Dunaliella*	Borowitzka, 1986.
7	Poly hydroxyl alkanoate, PHB	As a bioplastics	*Heloferax mediterranei, Halomonas boliviensis*	Fernandez-Castillo *et al.*, 1986;
8	Liposomes	Medicines and cosmetics	*Halobacterium cutirubrum*	Krishnan *et al.*, 2000, Sprott *et al.*, 2003
9	Bacteriocin and halocin	Medicines	*Haloferax mediterranii, Haloferax gibbonsii*	Platas *et al.*, 2002; Yun *et al.*, 2003
10	γ-D-glutamic acid)	biodegradable thickner, carrier of drug, food, pharmaceutical industry	*Natrialba* sp.	Hezayen *et al.*, 2000
11	Fermented foods	Preparation of "nam pla" (fermented fish sauce produced in Thailand), Development of aroma in sauce.	*Halobacterium sp., Halococcus sp.*	Thongthai and Siriwongpairat, 1990
12	Enzymes (amylases, nucleases, lipases, phosphatases, proteases etc.)	Food industries, pharmaceutical industries, detergent industries, leather industries, and many more	Halophilic archaea, bacteria and eukarya	Onishi and Hidaka, 1978; Onishi and Sonoda, 1979
13	Protein and peptides	feedstock for crab, shrimp, shellfish, chicken	*Dunaliella*	Galinski and Tindall, 1992).
14	Miscellaneous	Biodegration of oil pollution (Aromatic hydrocarbon)	*Haloferax* strain D1227	Oriel *et al.*, 1997
		Production of solar salt by rapid evaporation of water (Absorption of light due to red pigments)	*Dunaliella, Salinibacter*	Davis, 1974; Jones *et al.*, 1981
		Treatment of waste water	*Dunaliella*	Santos *et al.*, 2001

2.8 Enzymes from halophiles

Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. Many consumer products will increasingly benefit from the addition or exploitation of extremozymes. The extracellular hydrolytic enzymes like lipases, amylases, proteases, pullulanases, DNases and xylanases have quite diverse potential usage in different areas such as food industry, detergent industry, food and feed additives, biomedical sciences and chemical industries (Rao *et al.*, 1998)

2.8.1 Lipases

Lipases are the enzymes capable of catalyzing the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids. Lipases are an interesting group of enzymes used in detergent industries, food industries, pharmaceutical industries, paper-pulp industries etc. Lipases can be obtained from halophilic microorganisms like *Salinivibrio* sp. (Amoozegar *et al., 2008*), *Natronococcus* sp. (Boutaiba *et al.*, 2006), haloarchaeal strains (Ozcan *et al.*, 2009) etc.

2.8.2 Protease

Proteases are group of hydrolytic enzymes that degrade protein into peptides or amino acids. Bacterial proteases are most widely used as additives in laundry detergents, food processing, pharmaceuticals, leather and diagnostic reagents, waste management as well as silver recovery (Amoozegar *et al.*, 2007). Halophilic proteases have been purified and characterized from bacterial species including *Halobacillus* spp. (Karbalaei-Heidari *et al.*, 2009), *Bacillus* sp. (Kamekura and Onishi, 1974), *Salicola* sp. (Moreno *et al.*, 2009), *Pseudoaltermonas* sp. (Sanchez-Porro *et al.*, 2003), *Salinivobrio* sp. (Amoozegar *et al.*, 2007) etc.

2.8.3 Amylases

Starch is composed of D-glucose units. Starch is composed of two forms, amylose (15–25%), a linear polymer consisting of α-1, 4-linked glucopyranose residues, and amylopectin (75–85%), a branched polymer containing α-1, 6-linked branching points occurring at every 17–26 α-1, 4 glycosidic linkages. Starch degrading enzymes can be classified into two groups, endo-acting and exo-acting enzymes. Endoamylases (α–amylases) cleave interior linkages of starch randomly and yield linear and branched

oligosaccharides. Exoamylases hydrolyze the substrate from the non-reducing end. Amylases are widely used in baking industries, color and crumb softness, detergent industries, paper and pulp industries etc. (Gupta *et al.*, 2003). Amylase producing halophiles reported are *Halomonas meridiana* (Coronado *et al.*, 2000), *Halothermothrix orenii* (Tan *et al.*, 2008), *Streptomyces* sp. (Chakraborty *et al.*, 2009) etc.

2.8.4 Cellulase

Cellulase is hydrolytic enzyme having effect on cellulose, the most abundant plant biomass. Cellulases are classified into three groups: exoglucanases, endoglucanases and β–D–glucosidases. Exoglucanases cleave the cellobiosyl units from the non reducing ends of the cellulose chains. Endoglucanases hydrolyze the internal cellulosic linkages and β–D–glucosidases specifically cleave glucosyl units from the non-reducing ends of cello-oligosaccharides. Cellulases are used in textile industries, in detergent industries and bioremediation of cellulosic waste (Aygan and Arikan, 2008). Now a day, cellulases are widely used in fermentation industries for converting cellulose to fermentable sugar (Wang *et al.*, 2009). Halophilic cellulase can be derived from *Bacillus* sp. (Aygan *et al.*, 2008), *Salinivibrio* sp. (Wang *et al.*, 2009) etc.

2.8.5 Xylanases

Xylanases are the enzymes that degrade xylan. Xylanases are widely used in backing industries, paper pulping industries as biobleaching (Mamo *et al.*, 2009). Xylanases can be derived from halophilic bacteria like *Chromohalobacter* sp. (Prakash *et al.*, 2009) and *Nesterenkonia* sp. (Govender *et al.*, 2009).

2.8.6 Pectinases

There are basically three types of pectic enzymes

1. De–esterifying enzymes
2. Depolymerizing enzymes and
3. Protopectinases.

They can be further classified according to the following criteria; whether they cause random cleavage (endo-, liquefying or depolymerizing enzymes) or whether the cleavage is endwise (exo- or saccharifying enzymes) (Kashyap *et al.*, 2001).

Pectinases have been used in retting and degumming of fiber crops, textile processing, coffee and tea fermentations, paper and pulp industry, and oil extraction (Hoondal *et al*., 2002).

2.8.7 Chitinases

Chitinases are the group of enzymes used for degradation of chitin, a polymer present in exoskeleton of insects, invertebrates and cell wall of fungi. Chitinases can be divided into endo-chitinases and exo-chitinases on the basis of its mode of action. Chitinases are widely used in biodegradation of chitin, as a biocontrol agent in agriculture etc. *Planococcus rifitoensis* M2-26 (Badiaa *et al.,* 2009) is an efficient Chitinase producing moderate halophilic organism.

2.9 Applications of Lipase

Lipases are widely used in various fields like degradation of fatty acid waste (Masse *et al*., 2001), food processing, processing of fats and oils, cosmetics preparation, in pharmaceutical industries, paper industries, chemical industries etc. (Kazlauskas and Bornscheuer, 1998; Rubin and Dennis, 1997). Most of the lipases are derived from fungi and bacteria but halophilic lipases find its application because of their unique properties.

2.9.1 Lipase in food industries

Fat and oil plays an important role as a nutrient and source of energy in food. Lipases are used for the conversion of less desirable lipids to higher value lipids by modifying their properties by altering the location of fatty acid chains in the glyceride and replacing one or more of the fatty acids with new ones (Pabai *et al*., 1995a,b; Undurraga *et al*., 2001). Microbial lipases are used to obtain PUFAs (Poly Unsaturated Fatty Acids) which are essential for normal synthesis of lipid membranes and prostaglandins. PUFA are derived from animals and plant lipids by the action of microbial lipases. PUFAs are used as pharmaceuticals, neutraceuticals, and food additives (Gill and Valivety, 1997a; Belarbi *et al*., 2000). Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anti cholesterolemics, anti inflammatories, and thrombolytics (Gill and Valivety, 1997b; Belarbi *et al*., 2000). In addition, lipases have been used for

development of flavors in cheese ripening, bakery products, and beverages (Kazlauskas and Bornscheuer, 1998). Lipases are also used to remove fat from meat and fish products (Kazlauskas and Bornscheuer, 1998).

2.9.2 Lipase in detergent industries

Lipases are widely used as a detergent additive for the removal of fat from cloths. Lipases used for detergent additive must be able to hydrolyze any fat i.e. must have low substrate specificity, must be able to tolerate harsh washing conditions like extreme pH and temperature and must be stable against surfactant used in detergent. Lipases for above purposes and features can be obtained by screening (Yeoh *et al.*, 1986; Wang *et al.*, 1995) and protein engineering (Kazlauskas and Bornscheuer, 1998).

2.9.3 Lipase in pulp industries

Hydrophobic components (Pitch) like wax cause problems in paper and pulp manufacturing (Jaeger and Reetz, 1998). Pitch can be removed up to 90% by the application of lipase.

2.9.4 Lipase in oleochemical industry

Lipases are used widely in oleochemical industries for the minimizing thermal degradation during glycerolysis, alcoholysis and hydrolysis (Hoq *et al.*, 1985; Arbige and Pitcher, 1989). Currently immobilized lipases are used in place of emulsifier and organic solvents in oleochemical industries (Sonntag, 1984). Use of thermostable lipase in oleochemical industries has bright future (Macrae and Hammond, 1985).

2.9.5 Lipase in polymer synthesis

Lipases are widely useful in the reaction used for the synthesis of optically active polymers (Margolin, 1987). These polymers are used as an asymmetric reagents and absorbents. Lipases can also be used for the synthesis of biodegradable aromatic polyesters (Linko *et al.*, 1998).

2.9.6 Lipase in cosmetics

Vitamin A and its derivatives have great potential in cosmetics as skin care products. Water soluble Vitamin A derivatives are produced by the action of immobilized lipase (Maugard *et al.*, 2002). Lipases have also been used for hair waving preparation (Saphir, 1967).

2.9.7 Lipase in medical field

Lipases have been used as digestive aids since long. Many medical drugs contain hyaluronidase, thiomucase enzymes and lipases for use in skin inflammations (Berrobi *et al*, 1970). Lipases activate tumor necrosis factors and hence are used for the treatment of cancer (Kato *et al.*, 1989).

2.9.8 Lipase in bioremediation

Lipase derived from bacterial monoculture can be used for the bioaugmentation of lubricant contaminated water or soil (Vasileva, 2003).

2.9.9 Lipase as diagnostic tool

Lipases are used in the determination of serum triglycerides by colorimetric method. Serum lipase is an indicator of pancreatic disorder or its injury (Lott and Lu, 1991). Apart from all above applications, lipase from pathogenic bacteria is mainly used for the bio- typing of those pathogens. Lipases can also be used as a biosensor tool for diagnosis of disease. Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these functions as lipid biosensors (Karube and Sode, 1998) which can be used for blood cholesterol determination.

2.9.10 Lipase in leather industries

Lipases are used in leather industries for the removal of fat from it. Fat can be removed from bovine leather by the application of organic solvents or surfactants. Application of lipases for these purposes can minimize environmental pollution. Lipase gives uniform and cleaner leather and reduces fogging of leather. This is the main advantage of lipase in leather degreasing.

2.9.11 Miscellaneous uses

Lipases are used for the synthesis of biodiesel from vegetable oils (Shah *et al.*, 2004). Lipases can also be applied for the removal of fat layer from waste water for effective oxygen penetration during waste water treatment process (Bailey and Ollis, 1986). Lipase gives characteristics flavor development in black tea after formation of volatile compounds due to enzymatic breakdown of membrane lipid by lipase.

2.10 Isolation of halophiles

Lipase producing halophiles can be isolated from saline habitats like salt desert, crystalline pond, sea, esturine, aquatic animals and other salt containing habitats. Halotolerant, Moderate halophiles or extreme halophilic organisms can be isolated on different media containing salt. Lipase producing organisms can be isolated from the salt habitat contaminated with oil (Wang *et al.*, 1995)

2.11 Screening of extracellular enzyme producing organisms

Extracellular enzyme producing organisms can be screened on agar media containing respective substrates. Zone of substrate utilization can be considered as an indication of enzyme production.

2.11.1 Screening of lipase producing organisms

Screening of lipase producing organisms can mainly be performed on the medium containing tributyrene as a substrate. Lipase producing organisms can be detected on the basis of clear zone surrounding colony (Cardenas *et al.*, 2001). Lipase producing organisms can also be screened on medium containing Tween-80 and organisms give clear zone on medium (Sierra, 1957). They can also be screened on modified Rhodamine B agar (Wang *et al.*, 1995). Screening procedure can also be performed on N-agar containing olive oil and victoria blue as an indicator chromogenic substrate (Samad *et al.*, 1989; Martin *et al.*, 2003).

2.11.2 Screening of protease producing organisms

Protease producing halophiles can be screened on saline medium containing milk, NaCl supplemented with yeast extract and peptone (Ventosa *et al.*, 1982). Zone of precipitation of paracasein around the colonies on addition of specific reagents can be taken as evidence of proteolytic activity. Protease producing bacteria can also be

screened on the media containing 1% (w/v) Casein and same concentration of Milk powder (Siddalingeshwara *et al.*, 2010)

2.11.3 Screening of amylase producing organisms

Amylase producing organisms can be screened using starch agar medium containing NaCl. Clear zone surrounding colony after adding iodine solution is an indication of amylase production (Amoozegar *et al.*, 2003).

2.11.4 Screening of cellulase producing organisms

Cellulase producing organisms can be screened on medium containing 0.5% Carboxymethyl cellulose. Clear zone surrounding colony is an indication of enzyme production (Roxana cojoc *et al.*, 2009).

2.11.5 Screening of chitinase producing organisms

Chitinase producers can be screened on media containing chitin binding dye calcofluor white M2R in chitin agar. Microorganisms possessing high chitinolytic potential give a clear zone under ultraviolet light (Vaidya *et al.*, 2003).

2.12 Factors affecting lipase production

Lipase production is generally affected by pH, temperature, substrate concentration, dissolved oxygen etc. (Elibol and Ozer, 2001). Besides all above factors, it may also affected by availability of triglycerides, free fatty acids, hydrolysable esters, bile salts and glycerol which generally induces lipase production. Lipase production can be done in liquid media (Chisti, 1999) or solid media (Hemachander *et al.*, 2001). At larger scale, submerged culture medium is more effective for lipase production.

2.12.1 Effect of carbon source

Bacillus species are able to produce lipase in medium containing 1% olive oil but not in the medium without olive oil even after long incubation period (Sugihara *et al.*, 1991). Extracellular lipase production by *Rhodotorula glutinis* was investigated in case of two carbon sources and was compared, palm oil at a concentration of 2% was found to yield 12-fold more lipase than the fructose medium (Papaparaskevas *et al.*, 1992). Similarly, lipase from *Penicillium expansum* yielded maximum activity at 0.1% olive oil concentration at pH 8.3. Enzyme stability was enhanced by the addition

of Tween-20 and lubrol PX. The enzyme had a preference for triacylglycerols but showed no positional specificity (Sztajer *et al.*, 1993). It is clear that oil works as an inducer for lipase secretion. Lipase from *Pseudomonas pseudoalcaligenes* F-111 in a medium that contained both olive oil- 0.4% and TritonX-100- 0.2%. Surprisingly, addition of Triton X-100 in the medium enhanced lipase production by 50 fold as compared to medium containing only olive oil (Lin *et al.*, 1996). Lipase from *Bacillus thermoleovorans* ID-1 showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures. The organism could use olive oil as a sole source of carbon and produced lipase during the late exponential growth phase. The organism was able to utilize variety of lipoid substances like olive oil, soybean oil, mineral oils, tributyrene, Tween 20 etc. for lipase secretion (Lee *et al.*, 1999). Hence, it can be said that lipase is an inducible enzyme and requires presence of lipid for induction.

2.12.2 Effect of nitrogen sources

Lipase production from *Acremonium structum* required medium containing 35% (w/v) soybean meal as the nitrogen source (Okeke and Okolo, 1990). *Aspergillus oryzae* produced maximal lipase in a medium that was containing yeast extract 1%, peptone 2%, and soybean meal 3% as nitrogen sources (Ohnishi *et al.*, 1994). The enzyme was produced by using olive oil and tributyrene as substrates.

2.12.3 Effect of pH

pH largely affects production and activity of lipase. The initial pH of the growth medium is also important for lipase production. Maximum extracellular lipase activity was observed at alkaline pH for *P. fragi* (pH > 7) (Nashif and Nelson, 1953) and *P. aeruginosa* (pH 9.0) (Nadkarni, 1971). In contrary, some bacteria and fungi prefers to produce maximum lipase at acidic pH (4.0–7.0). This was found to be suitable for *M. caseolyticus* (Jonsson and Snygg, 1974) and *A. wentii* (Chander *et al.*, 1981).

2.12.4 Effect of temperature

Temperature is the environmental factor that affects growth and lipase production from all the organisms. Several researchers have investigated effect of temperature on growth and yield of lipase for different organisms. *Acinetobacter calcoaceticus* was

able to produce maximum lipase in mesophilic temperature range with optimum being 30˚C. (Mahler *et al.* 2000). *Acinetobacter calcoaceticus* LP009 was able to produce maximum lipase at lower temperature (15˚C as optimum) (Pratuangdejkul and Dharmsthiti 2000). Contrary, *Bacillus strain* A30-1 (ATCC 53841) was able to produce maximum lipase in thermophilic conditions i.e. 60˚C (Wang *et al.* 1995).

2.13 Purification of Lipase

The purification strategies employed for industrial scale purification of enzyme must be inexpensive, rapid, high-yielding and amenable to large-scale operations. It must yield continuous product recovery with fewer by-products. Purification of any enzyme is necessary for industrial purposes, understanding three dimensional structures and to determine structure-function relationship of proteins (Aires-Barros *et al.*, 1994). Researchers must determine optimal purification strategies for microbial lipases (Saxena *et al.*, 2003). Purification methods available for lipase are generally non-specific like precipitation, hydrophobic interaction, chromatography, gel filtration, and ion exchange chromatography. Affinity chromatography has been used in some cases to reduce the number of individual purification steps needed (Woolley and Peterson, 1994). An extracellular lipase from *Acetobacter calcoaceticus* BD 413 was purified to homogeneity using hydrophobic interaction FPLC (Fast Performance Liquid Chromatography) (Kok *et al.*, 1995). Lipase from *Pichia burtonii* was purified to homogeneity by a combination of DEAE-Sephadex A-50 ion exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing (Sugihara *et al.*, 1995). Extracellular lipase from *Rhizopus oryzae* can be purified by the combination of ammonium sulfate precipitation, sulfopropyl Sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl Sepharose chromatography step. Lipase was purified 1200 fold by above steps (Hiol *et al.*, 2000). A lipase from thermophilic *Bacillus* sp. was purified by ammonium sulfate and phenyl Sepharose column chromatography with 175 fold activity (Nawani and Kaur, 2000). Lipase from *P. aeruginosa* MB5001 was purified using a three-step procedure; concentration by ultrafiltration was followed by ion exchange chromatography and gel filtration (Chartrain *et al.* 1993). All the processes for purification of lipase are applied for small scale and little information is available for enzyme purification at larger scale.

2.14 Characterization of lipase

Lipase must be characterized for various parameters like pH optima, thermal stability, temperature optima, effect of inorganic salts etc.

2.14.1 pH optima of lipase

Lipase from halophilic bacteria is affected by change in pH and it having unique pH optima. Maximum lipase activity would be at neutral or alkaline pH, with the exception of lipase from *Pseudomonas fluorescens* SIK W1 that has an acidic optimum pH of 4.8. But in general, bacterial lipases are stable in a wide range of pH, from pH 4 to 11 (Gupta *et al.*, 2004). Lipase derived from halotolerant *Staphylococcus warneri* PB233 was stable between pH 5 to 12 with optimum activity at pH 7 (Werasit and Anan, 2007). Lipases from *Bacillus stearothermophillus* SB-1, *B. atrophaeus* SB-2 and *B.licheniformis* SB-3 were active over a broad range of pH from 3-12 (Bradoo *et al.*, 1999), where as lipase from *B. thermoleovorans* CCR11 was most active at pH 9-11 and was found to be stable at broad range of pH from 5-11 (Castro-Ochoa *et al.*, 2005). The purified lipase from marine *Vibrio fischeri* showed maximum stability at pH 8. At the pH values below 5, lipase lost about 40% of its activity after holding for 4 hrs. The pH range for stability was found to be 7 to 9 (Ranjitha *et al.*, 2009).

2.14.2 Thermostability of lipase

Thermal stability is a desirable characteristic of lipases (Janssen *et al.*, 1994). Bacterial lipases have been reported to have an optimum temperature in the range of 30 - 60°C. But there are some strains which have thermo-stability even at temperatures up to 100ºC (Gupta *et al.*, 2004). Lipase derived from halotolerant *Staphylococcus warneri* PB233 was stable between 30-80˚C with optimum activity at 40˚C (Werasit and Anan, 2007). Lipase of *V. fischeri* showed 80% stability at 35ºC, but the least residual activities was at 5 to 10˚C and 50ºC. No activity was observed in 60- 65ºC and above (Ranjitha *et al.*, 2009). The important thermostable enzyme from *Bacillus* had its maximum activity at 60˚ C and retained 100% of the original activity after being held at 75˚C for 30 min. The half-life of the enzyme was 8 h at 75˚C. The enzyme retained at least 90% of the original activity after being incubated at 60˚ C for 15 hour (Wang *et al.*, 1995). Industrial lipases must have such high thermostability in order to be used for various purposes. Similar type of thermostable lipases were

isolated from different bacterial species like, *B. stearothermophilus* (Kim *et al*., 1998); *Aeromonas sobria* (Lotrakul and Dharmsthiti, 1997); *Bacillus* sp. (Wang *et al*., 1995; Sidhu *et al*., 1998); *B. cereus* (El-Shafei and Rezkallah, 1997); *P. fluorescens* (Kojima *et al*., 1994); *Geotrichum* sp. (Macedo *et al*., 1997). Some reports shows increase in thermostability of lipase by the addition of stabilizer like sorbitol, glycerol and ethylene glycol (Nawani and Kaur, 2000).

2.14.3 Effect of inorganic salts on lipase activity

The effect of inorganic salts on lipase activity is either stimulatory or inhibitory. $CaCl_2$ is found to enhance enzyme activity and other salts have antagonistic effect (Ventos *et al*., 1998). But in general, the influence of different metal ions is specific and different for each type of bacterial lipases depending on the origin of the same (Bora and Kalita, 2004). Bacterial lipase from *Penicillium roqueforti* IAM7268 was not affected by Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, Cu^{2+}, EDTA, p-chloro mercuribenzoic acid, and iodoacetate (Mase *et al*., 1995). In contrast, the enzyme was inhibited by Ag^{+}, Fe^{2+}, Hg^{2+}, and isopropyl fluorophosphate. Lipases from *S.aureus and S. hyicus* were stimulated by Ca^{2+} and inhibited by EDTA (Dharmsthiti *et al*., 1998). Lipase of *P. aeruginosa* KKA-5 retained its activity in the presence of Ca^{2+} and Mg^{2+} but was slightly inhibited by Mn^{2+}, Cd^{2+}, and Cu^{2+}. Salts of heavy metals like Fe^{2+}, Zn^{2+}, Hg2 +, Fe^{3+} strongly inhibited the lipase, suggesting that they were able to alter the 3-D conformation of enzyme (Sharon *et al*., 1998).

CHAPTER-3

Materials and Methods

3.1. Sample Collection

A total of 10 samples of back waters of sea were collected from Little *Rann* of Kutch near Surajbari Bridge- Kutch, Gujarat, India [Latitude- 23°12'4.95"N and Longitude- 70°43'2.45"E] (Figure-3.1 A & B) including 5 salt samples, 2 soil (mud) samples and 3 water samples within 2 km. area. Samples of excreta of wild ass were also collected from "Indian wild ass sanctuary" near Dhrangadha, Dist- Surendranagar, Gujarat, India near little *Rann* of Kutch [Latitude- 22°98'4.181"N and Longitude- 71°51'0.242"E] (Figure-3.2). All the samples were characterized in terms of physical and chemical parameters.

Figure-3.1(A): site for the collection of sea samples (Red marks).

Fig-3.1 (B): site for the collection of sea samples (Red marks).

Figure-3.2 site for the collection of excreta samples (Red marks).

3.2. Enrichment and isolation of Organisms

Separate flasks with 50 ml sterile complete medium broth containing: (gm/100 ml) glucose 1; peptone 0.5; yeast extract 0.5; KH_2PO_4 1; Sodium Chloride 5-35; pH 7.2-7.4 and halophilic broth (Himedia) containing: (gm/100 ml) Casein acid hydrolysate-1; Yeast extract 1; Protease peptone-0.5; Trisodium citrate 0.3; Potassium chloride 0.2; Magnesium sulfate 2.5; Sodium chloride 5-35; pH 7.2-7.4.were added with a pinch of soil sample, salt sample and 3 ml of water sample.

Outer surface of wild ass excreta was scraped with sterilized knife in order to remove surface contaminants and then inoculated into 50 ml sterile complete medium broth containing: (gm/100 ml) glucose 1; peptone 0.5; yeast extract 0.5; KH_2PO_4 1; Sodium Chloride 5-15; pH 7.2-7.4 and halophilic broth (Himedia) containing: (gm/100 ml) Casein acid hydrolysate 1; Yeast extract 1; Protease peptone-0.5; Trisodium citrate 0.3; Potassium chloride 0.2; Magnesium sulfate 2.5; Sodium chloride 5-15; pH 7.2-7.4. Well isolated colonies were selected and pure cultures were obtained by subsequent streaking on agar plates. Total 30 isolates (extreme halophiles) were obtained from sea water and muddy soil samples and were designated from Ku-1 to Ku-30. Total 24 isolates (Moderate halophiles) were isolated from excreta of wild ass and designated from Mk-1 to Mk-24.

3.3. Maintenance of the Culture

The isolates were transferred on the slants (Complete medium, pH 7.2, salt concentration 15% w/v for extreme halophiles and 8% salt for moderate halophiles) and stored at 4^{o} C. The organisms were sub cultured monthly. All the 54 isolates were also preserved in glycerol stock culture.

3.4 Identification of the halophiles

All the 54 isolates were identified on the basis of Bergey's Manual of Systemic Bacteriology.

3.5 Characterization of organisms

The organisms were characterized in terms of colony morphology on halophilic agar containing: (gm/100 ml) Casein acid hydrolysate-1; Yeast extract 1; Protease peptone-0.5; trisodium citrate 0.3; Potassium chloride 0.2; Magnesium sulfate 2.5; Sodium

chloride 20 (For extreme halophiles) and 10 (For moderate halophiles); Agar-3; pH 7.2-7.4. Organisms were also characterized in terms of Gram's staining and capsules staining by bright field microscopy after activation in halophilic broth. Bile salt (Sodium deoxycholate, Himedia) tolerance in intestinal bacteria flora of wild ass was checked on halophilic agar containing bile salt. Intestinal bacteria were also studied on the basis of growth on MacConkey's agar and EMB agar.

3.6 Inoculum Preparation

For inoculum preparation, a loopful of culture from the culture suspensions prepared from pure cultures on slants was added in 25 ml sterile Halophilic broth and incubated on shaker for 24 hrs at 37^{O} C. 5 ml from this activated culture was then inoculated into 100 ml of sterile Halophilic broth (pH 7.2; salt 20% w/v for extreme halophiles and 10%w/v for moderate halophiles) and incubated for 24 h on shaker at 37°C.

3.7 Screening for lipase

Lipase producers were screened by using Tributyrene agar plates containing, (gm/ 100ml) Yeast extract- 1, NaCl- 10 (For moderate halophiles) 20 (For extreme halophiles), pH- 7.2, Agar- 3 and tributyrene- 1ml. Actively growing cultures of different isolates were inoculated on solid media by micropipette to perform spot test and plates were incubated for 3 days at 30^{O}C. After 3 days, plates were analyzed for clear zone surrounding the colony and zone index was calculated on the basis of ratio of zone diameter and colony diameter.

3.8 Screening for Amylase

For the screening of amylase producers, starch agar (gm/ 100ml): Starch- 0.2, Yeast extract- 0.5, Peptone- 1, NaCl- 10 (For moderate halophiles) 20 (For extreme halophiles), Agar- 3 and pH- 7.2 was used as a medium. Actively growing cultures of moderate and extreme halophiles were inoculated into medium by micropipette for spot test. All the plates were incubated for 2 days at 30° C. After incubation, iodine solution (gm/100ml: Iodine- 0.33, KI- 0.66) was added for the detection of clear zone surrounding the colony against blue background. Zone index was measured on the basis of ratio of zone diameter to colony diameter.

3.9 Screening for protease

Detection of protease production was carried out on Milk agar plates containing (gm/100ml) casein-3, peptone-1, yeast extract- 0.5, NaCl- 10 (For moderate halophiles) 20 (For extreme halophiles), Agar- 3 and gelatin agar medium containing (gm/ 100ml) Gelatin- 3, Peptone 1, NaCl 10 (For moderate halophiles) 20 (For extreme halophiles) and Agar- 3. The pH of the medium was adjusted to 7.2 by adding separately autoclaved 20% w/v Na_2CO_3. Actively growing cultures were inoculated on the medium as a spot. All the plates were incubated at 30˚C for 72 hrs. Protease production was monitored by adding Frazier's reagent.. Colonies showing clear zone in the surrounding area were considered to be protease producers. Zone index was measured on the basis of ratio of zone diameter to colony diameter.

3.10 Screening for Cellulase

Cellulase producing organisms were screened on Dubo's agar medium containing cellulose. Actively growing cultures were inoculated on Dubo's agar medium containing (gm/100 ml): Cellulose- 1, K_2HPO_4- 0.1, $NaNO_3$- 1, KCl- 0.05, $MgSO_4 \cdot 7H_2O$- 0.05, $FeSO_4 \cdot 7H_2O$- 0.001, Agar- 3, NaCl- 10 (For moderate halophiles) 20 (For extreme halophiles) and pH 7.2. All the plates were incubated at 30°C for 5 days. Zone index was measured on the basis of ratio of zone diameter to colony diameter.

3.11 Screening for Chitinase

For screening of Chitinase producers, Chitin agar plates containing: (gm/100 ml): Chitin- 1, Yeast extract- 0.5, Peptone- 0.5, NaCl- 10 (For moderate halophiles) 20 (For extreme halophiles), pH 7.2 and Agar- 3 were used. Actively growing cultures of Isolates were inoculated on the above plates and incubated for 72 hr at 37ºC, plates were observed for clear zone surrounding the colony.

3.12 Effect of salt, pH, temperature and substrate on Growth and Lipase Production

3.12.1 Effect of Salt

The effect of salt on growth and enzyme production by isolates was studied on Tributyrene agar plates with varying salt concentrations (10%-35% w/v for extreme halophiles and 10%-15% for moderate halophiles). After incubation for 72 hrs at 30°C, enzyme secretion was detected by clear zone surrounding the colonies.

3.12.2 Effect of pH

To monitor the effect of pH on growth and enzyme production, the pH of medium (Tributyrene agar plate) was adjusted by adding different amount of 1N HCl and 20% Na_2CO_3 (pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). The enzyme secretion was detected after 72 hrs at 30°C.

3.12.3 Effect of Temperature

The effect of temperature on growth and enzyme production was checked by incubating Tributyrene agar plates at different temperatures. The temperatures of the medium were adjusted to 20˚C, 30˚C, 40˚C, 50˚C, & 60˚C. The enzyme production was detected after 72 hrs at 30°C.

3.12.4 Effect of substrate concentration

The effect of substrate concentration on growth and enzyme production was checked by adding different amounts of substrate (Tributyrene- 1%, 2%, 3%, 4%, 5%, 6%) into the medium. After incubation, enzyme production was detected after 72 hrs at 30°C All the organisms were grown on medium containing olive oil as a substrate for the study of effect of salt, temperature and pH.

3.13 Effect of salt and pH on Growth and Amylase production

3.13.1 Effect of Salt

The effect of salt on growth and enzyme production by isolates was studied on Starch agar plates at varying salt concentrations (10%-35% w/v for extreme halophiles and 10%-15% for moderate halophiles). After incubation for 72 hrs at 30°C, enzyme secretion was detected by clear zone surrounding colony after adding iodine solution.

3.13.2 Effect of pH

To monitor the effect of pH on growth and enzyme production, the pH of medium (Starch agar plate) was set by adding different amounts of 1N HCl and 20% Na_2CO_3 (pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). The enzyme production was detected after 72 hrs.

3.13.3 Effect of Temperature

The effect of temperature on growth and enzyme production was checked by incubating Starch agar plates at different temperatures. The incubation temperatures were adjusted to 20˚C, 30˚C, 40˚C, 50˚C, 60˚C,. The enzyme production was detected after 72 hrs.

3.14 Enzyme assay for lipase

Lipase activity was determined as described by Pignede *et al,* by using olive oil as a substrate.

The assay:

The substrate emulsion was prepared with 50 ml olive oil and 100ml phosphate buffer. The reaction mixture contained 1 ml enzyme, 5 ml substrate and 2 ml of 50 mM phosphate buffer, pH 6.8 and was incubated for 1 hour at 37ºC with shaking. The reaction was stopped with 4 ml of acetone: ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50mM sodium hydroxide solution. One international unit of enzyme is defined as enzyme activity that produced 1µmole of fatty acid per min.

Formula:

Enzyme activity= Volume of alkali consumed × Normality of alkali /weight of Sample × time (min.)

3.16 Growth Kinetics with Reference to lipase Production

Activation of the culture was carried out in sterile complete media broth

Complete media broth

Tributyrene	1 ml
Peptone	1gm
Yeast extracts	0.5gm
Sodium chloride	10 gm (For moderate halophiles)
	15 gm (For extreme halophiles)
Distilled water	100 ml
pH	7.2

Growth and enzyme productivity at different time intervals was monitored. After every 8 hrs, samples were withdrawn aseptically from the growing culture and absorbance was measured at 540 nm. Graph of absorbance versus time was plotted. Similarly the enzyme activity was also measured from the sample to find optimum enzyme production.

3.17 Optimization of Medium for lipase production

3.17.1 Optimization of salt concentration:

Optimization of salt concentration for optimum enzyme production was carried out by adding varying NaCl concentrations in liquid media. NaCl concentration was maintained as 10%, 11%, 12%, 13%, 14%, and 15% for moderate halophiles and 10%, 15%, 20%, 25%, 30%, and 35% for extreme halophiles. Incubation period was followed by the measurement of enzyme activity.

3.17.2 Optimization of pH:

Optimization of pH for optimum enzyme production was carried out by setting varying pH values (adjusted by adding 1N HCl or 20% Na_2Co_3) in liquid media. Different pH values i.e. 4, 5, 6, 7, 8, 9, 10 were set for optimization.

3.17.3 Optimization of Temperature:

Temperatures were optimized by incubating media at 20˚C, 30˚C, 40˚C, 50˚C & 60˚C and were assayed by method discussed by Pignede *et al.*

3.17.4 Optimization of Substrate concentration:

Substrate concentration optimization was carried out by inoculating organisms into medium with different substrate (Tributyrene) concentration i.e. 1%, 2%, 3%, 4%, 5% and 6%. All the flasks were incubated in shaking condition at 30^{o}C followed by measurement of enzyme activity.

3.18 Amylase assay

Enzyme assay for amylase was carried out by Dinitrosalicylic acid (DNSA) method using starch as the substrate. The enzyme (0.5ml) was added to 0.5ml 1% starch solution. The reaction was incubated at 30^{o} C for 10 min and then the enzyme reaction

was terminated by the addition of 1.0 ml Dinitrosalycilic acid reagent (1g, Dinitrosalicylic acid; 1.6 g, NaOH; 30g, Sodium potassium tartarate; D/W, 100ml). After termination, the reaction tube was kept in boiling water bath for 10 min. The reaction mixture was diluted up to 10 ml by addition of D/W and the absorbance was measured at 540 nm. One unit of amylase was defined as the amount of enzyme liberating 1 µg of maltose per minute under the assay conditions. Enzyme units were measured using standard maltose (100-1000 µg).

3.19 Effect of pH, temperature, nitrogen source and substrate concentration on growth and amylase production

3.19.1 Effect of pH on growth and amylase production

For the investigation of effect of pH on amylase production in liquid media, starch broth was prepared containing: (gm/ 100ml): Starch- 0.5, Yeast extract- 0.5, Peptone- 1, and NaCl- 10. Organisms screened on solid starch agar plates were inoculated into medium containing pH-5, 6, 7, 8, and 9. pH was adjusted by adding either 1N HCl or 20% Na_2Co_3. All the flasks were incubated at 30˚ C in shaking condition and biomass and enzyme activity were measured at an interval of 24 hrs.

3.19.2 Effect of temperature on growth and amylase production

To check optimum temperature for halophilic amylase, starch broth containing: (gm/ 100ml): Starch- 0.5, Yeast extract- 0.5, Peptone- 1, NaCl- 10, pH-6. All the flasks were inoculated with actively growing cells as inoculum. 5% inoculum was added into assay medium. Incubation was performed at different temperature i.e. 25˚C, 37˚C, and 50˚C followed by measurement of biomass and enzyme activity at an interval of 24 hrs.

3.19.3 Effect of nitrogen sources on growth and amylase production

Effect of Nitrogen Sources on growth and amylase production was studied using different organic and inorganic nitrogen sources like Peptone, glycine, ammonium sulfate and urea at a concentration of 1 % (w/v). Additionally, medium also contained: (gm/100ml) Starch- 0.5, Yeast extract- 0.5, NaCl- 10, pH-6. The flasks

were incubated at 37˚C, biomass and enzyme activity were measured at an interval of 24 hrs.

3.19.4 Effect of starch concentration on growth and amylase production

Effect of starch concentration on growth and amylase production was studied using different starch concentrations (0.3%, 0.6%, 0.9%, 1.2% and 1.5%) and medium containing: (gm/100ml) Yeast extract- 0.5, NaCl- 10, peptone- 1, pH-6. The flasks were incubated at 37˚C, biomass and enzyme activity were measured at an interval of 24 hrs.

3.20 Substrate kinetics (Km and Vmax) for amylase

Substrate kinetics i.e. Km and Vmax were determined for crude amylase on substrate starch. The enzyme assay was carried out at various concentrations of starch in the range of 2-10 mg/ml. Enzyme activity was determined by DNSA method using maltose (100-1000 µg) as a standard. The Km and Vmax were determined on the basis of substrate curve.

3.21 Partial Purification of lipase

The crude lipase was fractioned by ammonium sulphate precipitation. Various fractions were collected i.e. 20%, 40%, 60%, 80% and 100%. After addition of particular amount of ammonium sulphate, solution in the flasks was stirred on magnetic stirrer. Then it was placed in refrigerator for overnight. Precipitated fractions were collected by centrifugation of broth at 10,000 rpm at 4˚C for 10 mins. The fractions were dissolved in little amount of phosphate buffer and dialyzed by dialysis membrane (Himedia) at 4˚C for overnight. Dialyzed enzyme was used as a source of crude enzyme. All the fractions were analyzed in terms of protein content by Folin's method and amount of lipase by Pignede's method.

3.22 Characterization of lipase

3.22.1. Temperature optima of lipase

In order to determine temperature optima of lipase, reaction mixture was incubated at different temperatures. The range of temperature was 20˚C to 70°C. Lipase activity was determined by the Pignede's method as described earlier.

3.22.2 Thermal stability

Thermal stability of lipase was determined by incubating enzyme at 60˚C and 70˚C for 1 hour and 2 hour followed by rapid cooling at 4˚C. The enzyme activity was performed by Pignede's method and residual activity was calculated from available data.

3.22.3 Effect of pH on lipase

The effect of pH on lipase was determined by preparing the substrate emulsion in various buffers with varying pH. Buffers were Citrate phosphate buffer (pH 5-7); Tris-HCl buffer (pH 8-9); Glycine-NaOH buffer (pH 9-10); NaOH-Borax buffer (pH 10-11). After incubation of reaction mixture for 1 hour in shaking condition, lipase activity was determined.

3.22.4 Effect of inorganic salts on lipase

Effect of inorganic salts on lipase activity was determined by incubating enzyme at 30˚C for 1 hour followed by determination of enzyme activity. Inorganic salts were NaCl (10 Mm), $BaCl_2$ (0.001 M), $MgCl_2$ (0.001 M), KCl (2 Mm), $FeSo_4$ (0.001 M), $CaCl_2$ (0.001 M), NaF (2 Mm), $MnCl_2$ (2 Mm), Ethylene diamine tetra acetic acid (0.5%), Sodium dodecyl sulphate (0.5%). To determine effect of inorganic salts on lipase activity, it was compared with control containing no inorganic salts.

3.22.6 Effect of urea on lipase denaturation

Urea was used as denaturant at 8 M. The partially purified lipase was incubated for 1 hour and 2 hour (24 hours for one organism). After incubation, enzyme activity was determined by method discussed and residual activity was calculated by comparison with control containing no urea.

3.23 UV survival curve for halophiles

After activation of organisms on complete media broth, biomass of an organism was obtained by centrifugation at 8,000 rpm for 10 mins followed by resuspending in sterile N-saline solution. The organisms were exposed to UV radiation for different

time interval (30 sec. to 120 sec.) at the distance of 30 cm. UV exposed cells were serially diluted up to 10^{-7}. Appropriate diluted samples were plated on Complete medium agar containing (gm/100 ml) Glucose-1; Peptone-1; Yeast extract- 0.5; Sodium chloride- 10 (For moderate halophiles) and 15 (For extreme halophiles); pH-7.2. After incubation at 30˚C for 48 hours, colonies of each plate were counted and no. of organisms was calculated on the basis of following formula.

$$N=Y/VX$$

Where, N=No. of organisms,
Y= No. of colonies,
V=Volume of aliquote,
X=Dilution

3.24 UV Mutagenesis

Activated cultures of halophiles were taken in sterile empty 10 ml centrifuge tube followed by centrifugation at 8,000 rpm for 10 mins. After centrifugation, supernatants were discarded and pellets were redissolved into sterile N-saline solution. The cultures was then taken into sterile petriplates and was exposed to UV radiation for 90 sec. at the distance of 30 cm. Exposed cultures tubes were wrapped with carbon paper in order to prevent photoreactivation. Then UV exposed and unexposed cultures were inoculated into medium containing 1% Tributyrene. All the media were incubated at particular time period on shaking condition followed by measurement of enzyme activity.

3.25 Molecular identification of selected isolates

Molecular identification (16's r-RNA sequencing) was performed for Mk-4, Mk-18, Mk-24, Ku-10, Ku-19 and Ku-20 at Gujarat State Biotechnology Mission (GSBTM). Bioinformatics tools were used to construct dandogram.

CHAPTER-4

Results

- Samples collected from different sampling sites including water, salt, mud and excreta samples vary in their physical appearance like colour, texture, moisture content and in microbial variety.
- Halophiles from wild ass excreta could tolerate 5-15%, NaCl w/v. However, isolates obtained from water, salt and mud samples could tolerate NaCl up to 35%, w/v

Table-4.1 Characterization of samples

S.No.	Collection site	Appearance	Color	Isolated organisms
M-1	2 km form Surajbari ri bridge, towards east	Salt sample, Granular, Slightly wet	White	Ku-1, Ku-2, Ku-3
M- 2	1 km. from Surajbari ri bridge, towards north	Salt sample, Granular, Very wet	Off-white	Ku-4, Ku-5
M-3	1.5 km from Surajbari ri bridge, towards north	Mud sample, Sticky	Black	Ku-6, Ku-7, Ku-8
M-4	1.5 km from Surajbari ri bridge, towards north	Salt sample, Granular, Very wet	Off-white	Ku-9, Ku-10, Ku-11, Ku-12
M-5	Under surajbari bridge	Salt sample, Granular, Slightly wet	Off-white	Ku-13, Ku-14, Ku-15
M-6	Under the Surajbari ri bridge	Mud sample, Sticky, Wet (mud with water)	Black	Ku-16, Ku-17, Ku-18
M-7	1 km from Surajbari ri bridge, towards north	Salt sample, Granular, Wet with high amount of moisture.	Blackish-white	Ku-19, Ku-20, Ku-21, Ku-22
M-8	Near Surajbari bridge, towards north	Water sample, Clear water with no turbidity	Clear	Ku-23, Ku-24, Ku-25
M-9	Near Dhrangadhra (Dist.-Surandranagar)	Excreta of wild ass, Dry with no moisture	Black	Mk-1 to Mk-24.
M-10	Under Surajbari bridge	Water sample, High turbidity	Highly turbid	Ku-28, Ku-29, Ku-30
M-11	1 km from Surajbari ri bridge, towards north	Water sample, water with little turbidity	Slight turbid	Ku-26, Ku-27

- Total 24 moderate halophiles were obtained from wild ass excreta samples (Mk-1 to Mk-24) and 30 extreme halophiles were isolated from other samples (Ku-1 to Ku-30) as indicated in Table-4.1
- All the isolates have diversity in colony characteristics (Table- 4.2 to 4.8) and also diversified cell morphology on the basis of Gram's staining (Figure- 4.1).

Table- 4.2 Colony characteristics of moderate halophiles (Mk-1 to Mk-8)

Isolates→ / Colony characters↓	Mk-1	Mk-2	Mk-3	Mk-4	Mk-5	Mk-6	Mk-7	Mk-8
Size	Big	Small	Mediu	Big	Mediun	Medium	Medium	Small
Shape	Irregular	Round	Round	Round	Round	Irregular	Round	Round
Margin	Irregular	Entire	Entire	Entire	Entire	Undulate	Entire	Entire
Elevation	Flat	Slightly Raised	Flat	Slightly Raised	Flat	Flat	Slightly Raised	Slightly Raised
Texture	Smooth	Smooth	Rough	Rough	Rough	Smooth	Rough	Rough
Consistency	Sticky	Sticky	Sticky	Powdery	Sticky	Sticky	Powdery	Powdery
Pigmentation	White	White	White	White	White	White	White	White
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.3 Colony characteristics of moderate halophiles (Mk-9 to Mk-16)

Isolates→ / Colony characters↓	Mk-9	Mk-10	Mk-11	Mk-12	Mk-13	Mk-14	Mk-15	Mk-16
Size	Big	Medium	Medium	Small	Medium	Big	Medium	Small
Shape	Round	Round	Irregular	Round	Irregular	Round	Irregular	Round
Margin	Entire	Entire	Irregular	Entire	Irregular	Entire	Irregular	Entire
Elevation	Flat	Flat	Flat	Raised	Slightly raised	Raised	Flat	Raised
Texture	Smooth	Smooth	Smooth	Rough	Smooth	Rough	Rough	Smooth
Consistency	Sticky	Sticky	Sticky	Powdery	Sticky	Rigid	Powdery	Sticky
Pigmentation	White	White	White	White	White	White	White	White
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.4 Colony characteristics of moderate halophiles (Mk-17 to Mk-24)

Isolates→ / Colony characters↓	Mk-17	Mk-18	Mk-19	Mk-20	Mk-21	Mk-22	Mk-23	Mk-24
Size	Small	Small	Medium	Medium	Medium	Medium	Medium	Small
Shape	Round	Round	Round	Round	Round	Round	Round	Round
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Flat
Texture	Smooth	Smooth	Rough	Smooth	Smooth	Smooth	Rough	Rough
Consistency	Sticky	Rigid	Rigid	Sticky	Sticky	Sticky	Sticky	Powdery
Pigmentation	White	White	White	White	White	White	White	White
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.5 Colony characteristics of extreme halophiles (Ku-1 to Ku-8)

Isolates→ / Colon characters↓	Ku-1	Ku-2	Ku-3	Ku-4	Ku-5	Ku-6	Ku-7	Ku-8
Size	Big	Medium	Small	Medium	Big	Medium	Small	Medium
Shape	Round	Round	Irregular	Round	Irregular	Round	Round	Irregular
Margin	Entire	Entire	Irregular	Irregular	Irregular	Irregula	Entire	Irregular
Elevation	Raised	Slightly Raised	Flat	Raised	Slightly Raised	Flat	Raised	Slightly Raised
Texture	Rough	Rough	Rough	Rough	Smooth	Smooth	Smooth	Rough
Consistency	Sticky	Powdery	Powdery	Powdery	Sticky	Rigid	Rigid	Sticky
Pigmentation	White	Yellowis	Yellowish	Pink	Pink	White	White	Yellowish
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.6 Colony characteristics of extreme halophiles (Ku-9 to Ku-16)

Isolates→ / Colony characters	Ku-9	Ku-10	Ku-11	Ku-12	Ku-13	Ku-14	Ku-15	Ku-16
Size	Medium	Big	Small	Medium	Big	Big	Big	Medium
Shape	Irregular	Round	Round	Irregular	Irregular	Round	Round	Round
Margin	Irregular	Entire	Entire	Irregular	Irregular	Irregula	Irregular	Entire
Elevation	Flat	Raised	Flat	Flat	Flat	Raised	Flat	Flat
Texture	Rough	Smooth	Rough	Rough	Smooth	Smooth	Smooth	Rough
Consistency	Powdery	Sticky	Rigid	Rigid	Powdery	Rough	Slimy	Powdery
Pigmentation	White	White	White	Pink	Pink	White	White	White
Opacity	Trans-lucent	Trans-lucent	Trans-lucen	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.7 Colony characteristics of extreme halophiles (Ku-17 to Ku-24)

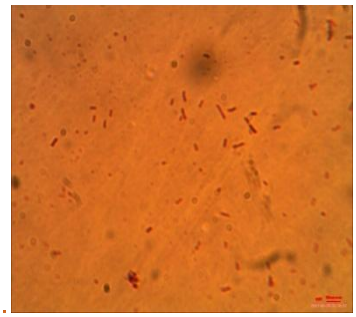
Isolates→ / Colon characters↓	Ku-17	Ku-18	Ku-19	Ku-20	Ku-21	Ku-22	Ku-23	Ku-24
Size	Medium	Small	Big	Small	Big	Medium	Small	Medium
Shape	Irregular	Irregular	Round	Round	Irregular	Irregular	Round	Round
Margin	Irregular	Irregular	Entire	Entire	Irregular	Irregular	Entire	Entire
Elevation	Slightly Raised	Raised	Flat	Raised	Slightly Raised	Slightly Raised	Flat	Raised
Texture	Smooth	Rough	Smooth	Smooth	Smooth	Smooth	Rough	Smooth
Consistency	Slimy	Rigid	Slimy	Slimy	Slimy	Slimy	Powdery	Rigid
Pigmentation	Pink	Pink	White	White	White	White	White	White
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Table- 4.8 Colony characteristics of extreme halophiles (Ku-25 to Ku-30)

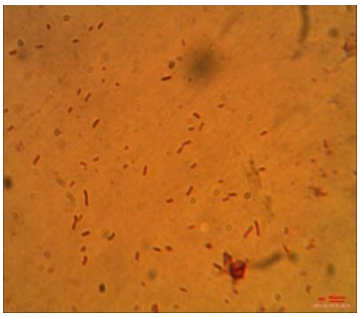
Isolates→ / Colony characters↓	Ku-25	Ku-26	Ku-27	Ku-28	Ku-29	Ku-30
Size	Small	Big	Small	Big	Big	Medium
Shape	Irregular	Round	Irregular	Irregular	Irregular	Round
Margin	Irregular	Entire	Irregular	Irregular	Irregular	Entire
Elevation	Raised	Slightly Raised	Flat	Flat	Raised	Slightly Raised
Texture	Rough	Rough	Smooth	Rough	Rough	Rough
Consistency	Rigid	Powdery	Slimy	Powdery	Rigid	Rigid
Pigmentation	Pink	White	White	Pink	White	Pink
Opacity	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent	Trans-lucent

Figure- 4.1 Gram's Staining of Isolates

Mk-1



Mk-2



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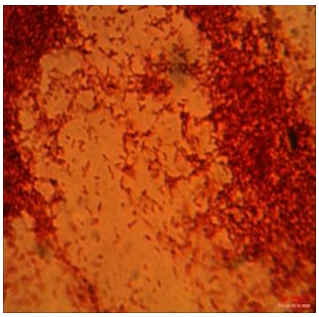

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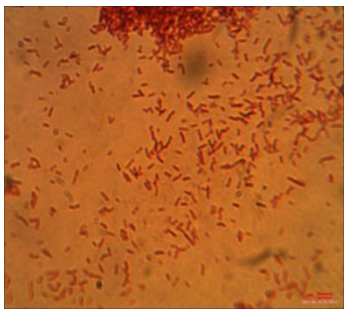

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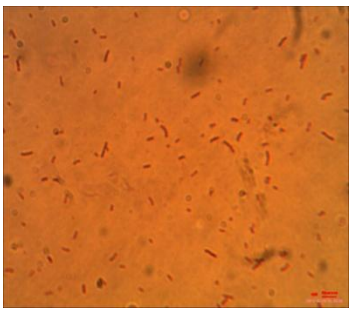

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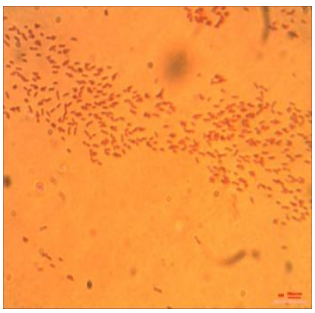

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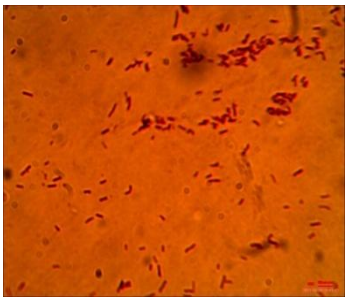

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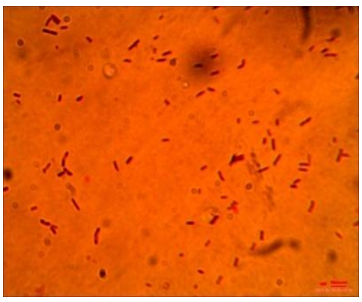

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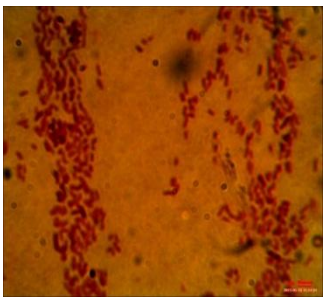

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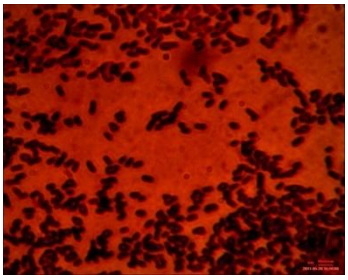

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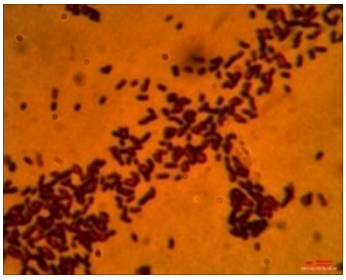

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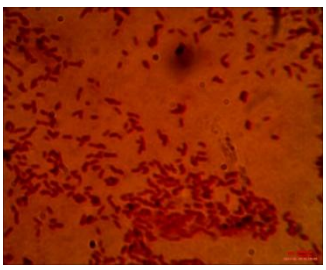

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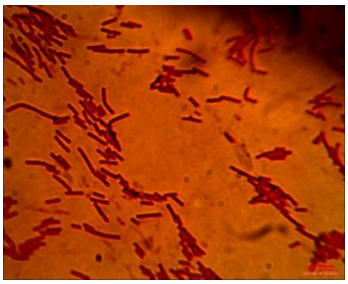

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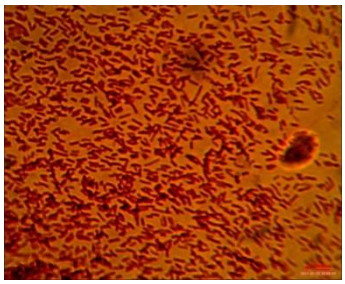

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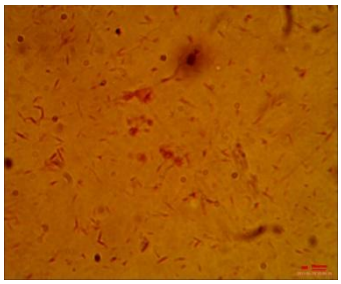

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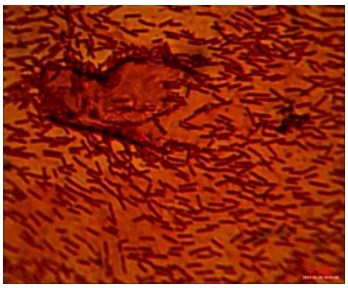

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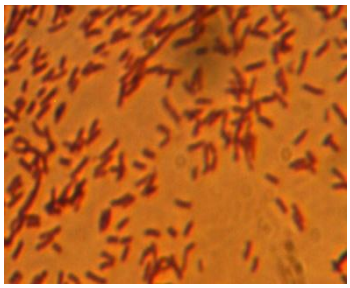

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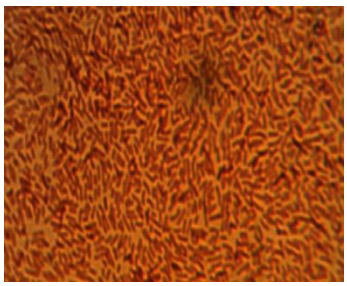

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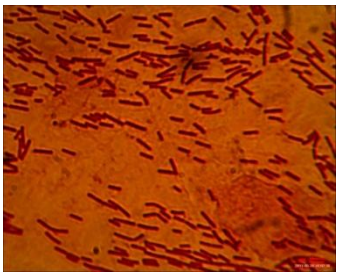

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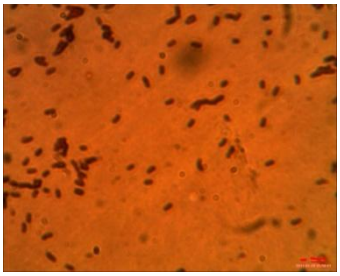

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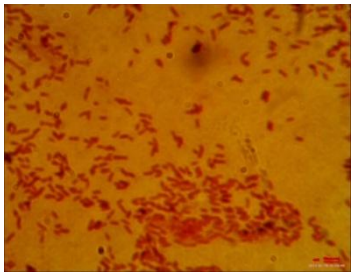

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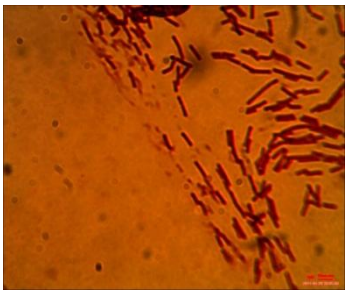

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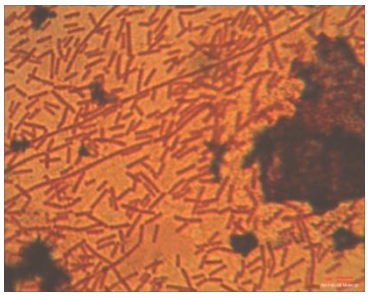

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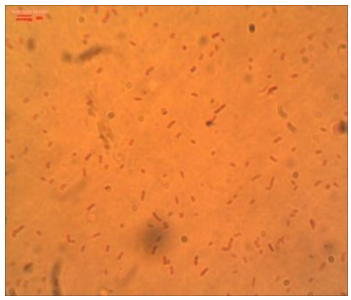

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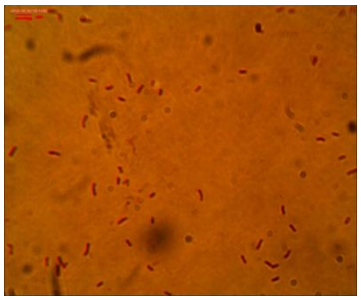

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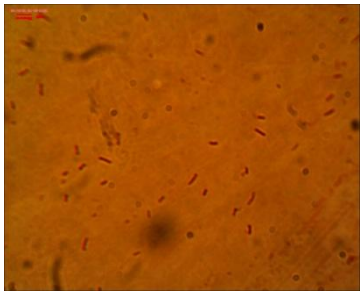

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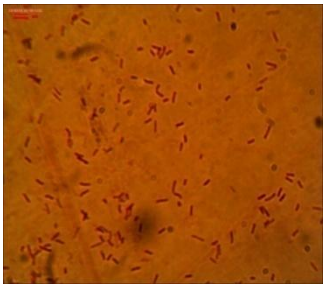

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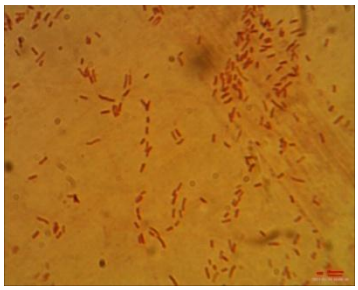

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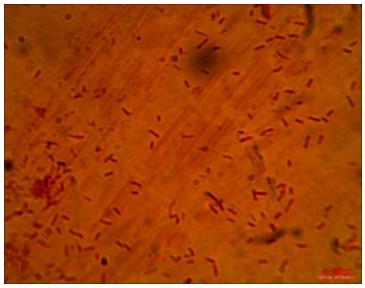

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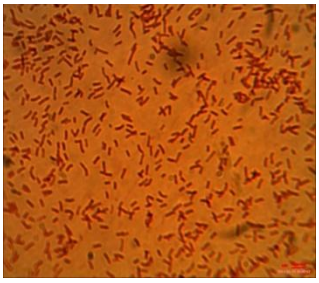

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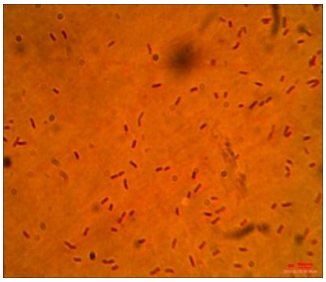

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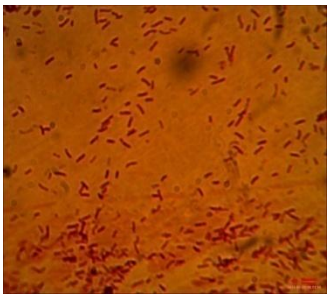

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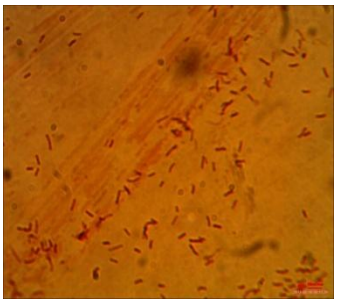

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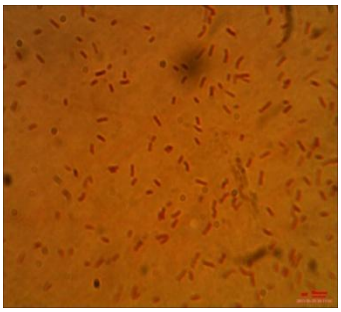

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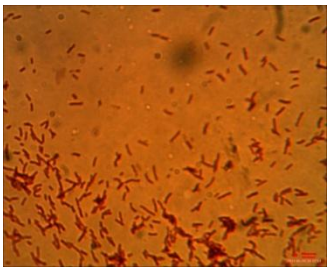

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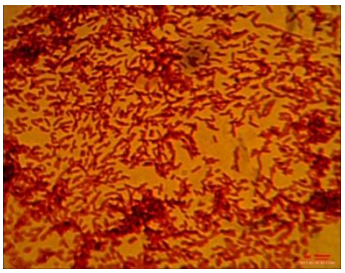

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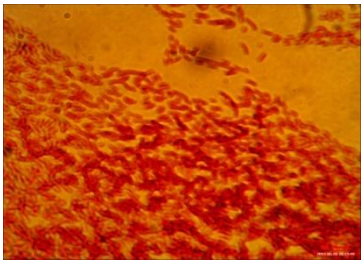

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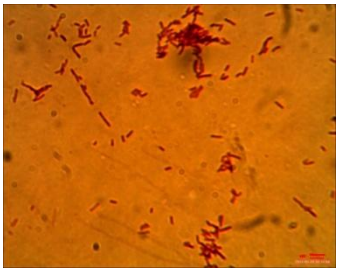

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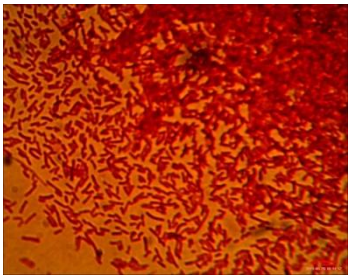

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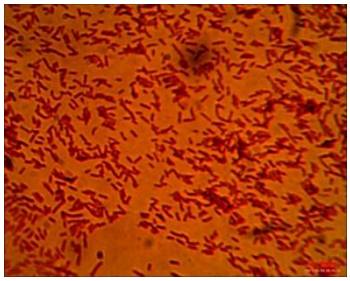

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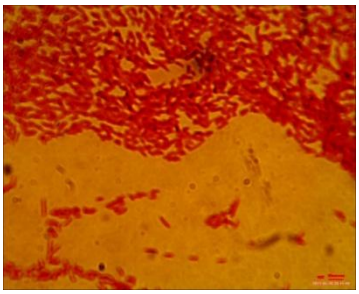

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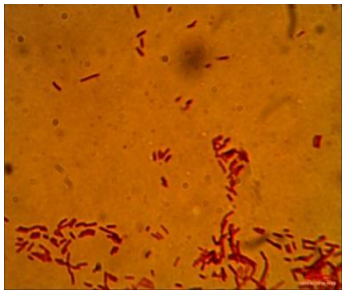

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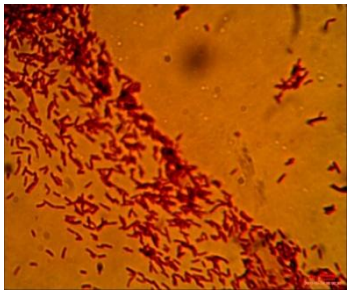

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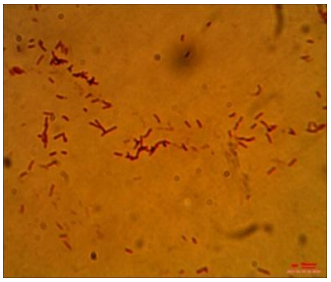
Ku-23



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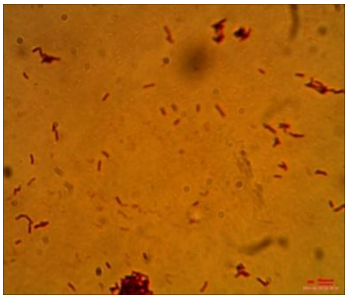

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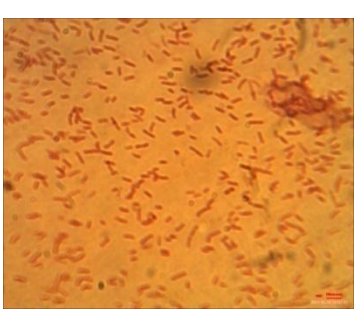
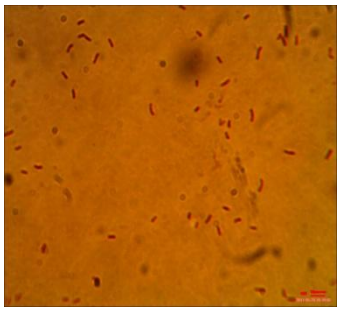
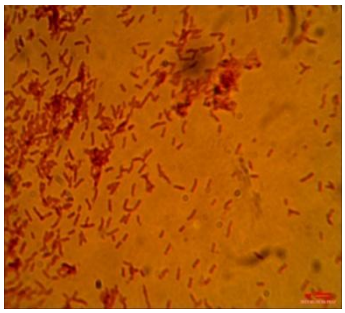

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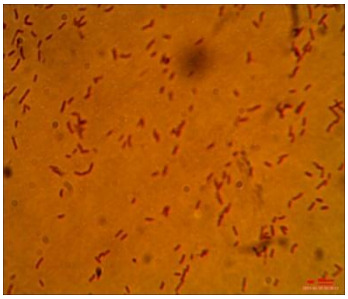
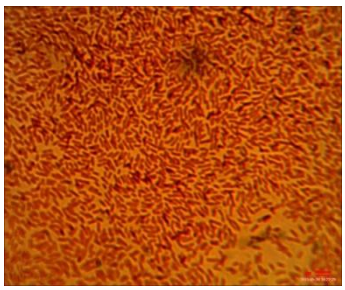
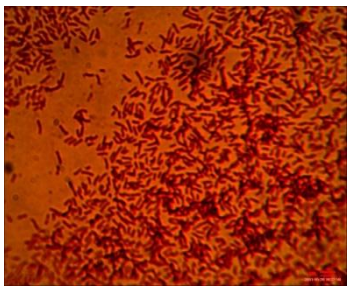




- Results of biochemical tests performed for the identification of the isolates as per 9th edition of Bergey's Manual of Systematic Bacteriology showed different biochemical and morphological properties
- As is indicated in Table-4.9 to 4.19, differences in their biochemical reactions were significant.

Table- 4.9 Result of Biochemical Tests for Samples - (Mk-1 to Mk-5)

Sr. No.	Name of the Test	Mk-1	Mk-2	Mk-3	Mk-4	Mk-5
1	Gram's stain	+	+	Variable	Variable	+
2	Capsule Stain	-	-	-	-	-
3	Cell shape	Long rods	Long rods	Long rods	Long rods	Long rods
4	Methyl Red test	+	-	-	-	-
5	Voges-Proskaur test	+	-	-	-	-
6	Starch hydrolysis	-	-	-	-	-
7	Casein hydrolysis	+	-	-	+	-
8	Gelatin hydrolysis	+	-	-	+	-
9	Citrate utilization	-	+	+	-	-
10	Indole production	-	-	-	-	-
11	Motility	+	-	+	+	-
12	Catalase	+	+	+	+	+
13	Phenyl- alanine deaminase	-	-	-	-	-
14	Nitrate reduction	+	+	+	+	-
15	Growth on Nacl					
	2% NaCl	-	-	+	-	-
	5% NaCl	+	+	+	+	+
	7% NaCl	+	+	+	+	+
	9% NaCl	+	+	+	+	+
16	Growth on pH					
	pH 5	+	+	+	+	+
	pH 9	+	+	+	+	-
17	Urease	-	-	-	-	-
18	Growth at Temperature					
	4°C	-	+	-	-	-
	37°C	+	+	+	+	+
	50°C	+	+	+	+	+
19	Acid production from					
	Glucose	+	+	+	+	-
	Maltose	-	-	+	-	-
	Fructose	+	+	+	+	-
	Sucrose	-	+	+	+	-
	Xylose	-	+	+	+	-
20	Growth on MacConky's agar	-	-	-	-	-
21	Growth on EMB agar	-	-	-	-	-
22	Bile salt tolerance	+	+	+	+	+

Table- 4.10 Result of Biochemical Tests for Samples- (Mk-6 to Mk-10)

Sr. No.	Name of the Test	Mk-6	Mk-7	Mk-8	Mk-9	Mk-10
1	Gram's stain	Variable	+	+	Variable	+
2	Capsule Stain	-	-	-	-	-
3	Cell shape	Long rods	Long rods	Long rods	Long rods	Long rods
4	Methyl Red test	-	+	-	-	-
5	Voges-Proskaur test	-	-	-	-	-
6	Starch hydrolysis	+	+	-	+	-
7	Casein hydrolysis	+	+	-	-	-
8	Gelatin hydrolysis	+	+	-	-	-
9	Citrate utilization	-	-	+	-	-
10	Indole production	-	-	-	-	-
11	Motility	-	+	+	+	+
12	Catalase	+	+	+	+	+
13	Phenyl- alanine deaminase	-	-	-	-	-
14	Nitrate reduction	+	+	+	-	-
15	Growth on Nacl					
	2% NaCl	-	-	+	-	+
	5% NaCl	+	-	+	-	+
	7% NaCl	+	+	+	+	+
	9% NaCl	+	+	+	+	+
16	Growth on pH					
	pH 5	+	+	+	+	+
	pH 9	+	-	+	+	-
17	Urease	-	-	-	-	-
18	Growth at Temperature					
	4°C	-	-	-	-	-
	37°C	+	+	+	+	+
	50°C	+	+	+	+	+
19	Acid production from					
	Glucose	+	+	-	+	-
	Maltose	+	+	-	+	-
	Fructose	+	-	-	+	-
	Sucrose	+	+	-	-	-
	Xylose	+	-	-	+	-
20	Growth on MacConky's agar	-	-	-	-	-
21	Growth on EMB agar	-	-	-	-	-
22	Bile salt tolerance	+	+	+	+	+

Table- 4.11 Result of Biochemical Tests for Samples - (Mk-11 to Mk-15)

Sr. No.	Name of the Test	Mk-11	Mk-12	Mk-13	Mk-14	Mk-15
1	Gram's stain	+	Variable	Variable	Variable	Variable
2	Capsule Stain	-	-	-	-	-
3	Cell shape	Long rods	Long rods	Long rods	Long rods	Long rods
4	Methyl Red test	-	-	-	-	-
5	Voges-Proskaur test	-	-	-	-	-
6	Starch hydrolysis	-	+	-	-	+
7	Casein hydrolysis	+	+	-	-	+
8	Gelatin hydrolysis	+	+	-	-	+
9	Citrate utilization	+	-	-	-	-
10	Indole production	-	-	-	-	-
11	Motility	-	+	-	+	+
12	Catalase	+	+	+	+	+
13	Phenyl-alanine deaminase	-	-	-	-	-
14	Nitrate reduction	-	-	-	-	+
15	Growth on Nacl					
	2% NaCl	-	+	-	-	-
	5% NaCl	+	+	+	+	+
	7% NaCl	+	+	+	+	+
	9% NaCl	+	+	+	+	+
16	Growth on pH					
	pH 5	+	+	+	+	+
	pH 9	+	+	+	+	+
17	Urease	-	-	-	-	
18	Growth at Temperature					
	4°C	-	-	-	-	-
	37°C	+	+	+	+	+
	50°C	+	+	+	+	-
19	Acid production from					
	Glucose	+	-	-	+	+
	Maltose	+	-	-	-	+
	Fructose	-	-	-	-	+
	Sucrose	-	-	-	-	-
	Xylose	+	-	-	-	+
20	Growth on MacConky's agar	-	-	-	-	-
21	Growth on EMB agar	-	-	-	-	-
22	Bile salt tolerance	+	+	+	+	+

Table- 4.12 Result of Biochemical Tests for Samples- (Mk-16 to Mk-20)

Sr. No.	Name of the Test	Mk-16	Mk-17	Mk-18	Mk-19	Mk-20
1	Gram's stain	Variable	Variable	Variable	Variable	+
2	Capsule Stain	-	-	-	-	-
3	Cell shape	Long rods	Long rods	Long rods	Long rods	Long rods
4	Methyl Red test	+	+	-	-	-
5	Voges-Proskaur test	+	-	-	-	-
6	Starch hydrolysis	+	+	-	+	-
7	Casein hydrolysis	-	+	+	+	-
8	Gelatin hydrolysis	-	+	+	+	-
9	Citrate utilization	+	+	-	-	-
10	Indole production	-	-	-	-	-
11	Motility	+	+	+	-	+
12	Catalase	+	+	+	+	+
13	Phenyl- alanine deaminase	-	-	-	-	-
14	Nitrate reduction	+	-	+	-	+
15	Growth on Nacl					
	2% NaCl	-	-	-	+	-
	5% NaCl	+	+	+	+	+
	7% NaCl	+	+	+	+	+
	9% NaCl	+	+	+	+	+
16	Growth on pH					
	pH 5	+	+	+	+	+
	pH 9	+	+	+	+	+
17	Urease	-	-	-	-	-
18	Growth at Temperature					
	4°C	-	-	-	-	-
	37°C	+	+	+	+	+
	50°C	+	+	+	-	+
19	Acid production from					
	Glucose	+	+	+	+	-
	Maltose	-	+	-	-	-
	Fructose	+	+	+	-	-
	Sucrose	+	+	-	+	-
	Xylose	+	+	+	+	-
20	Growth on MacConky's agar	-	-	-	-	-
21	Growth on EMB agar	-	-	-	-	-
22	Bile salt tolerance	+	+	+	+	+

Table- 4.13 Result of Biochemical Tests for Samples - (Mk-21 to Mk-24)

Sr. No.	Name of the Test	Mk-21	Mk-22	Mk-23	Mk-24
1	Gram's stain	Variable	+	+	+
2	Capsule Stain	-	-	-	-
3	Cell shape	Long rods	Long rods	Long rods	Long rods
4	Methyl Red test	-	-	-	-
5	Voges-Proskaur test	-	-	-	-
6	Starch hydrolysis	+	-	-	-
7	Casein hydrolysis	-	-	+	+
8	Gelatin hydrolysis	-	-	+	+
9	Citrate utilization	-	-	-	-
10	Indole production	-	-	-	-
11	Motility	+	-	-	+
12	Catalase	+	+	+	+
13	Phenyl- alanine deaminase	-	-	-	-
14	Nitrate reduction	-	-	+	+
15	Growth on Nacl				
	2% NaCl	-	-	-	-
	5% NaCl	+	+	+	+
	7% NaCl	+	+	+	+
	9% NaCl	+	+	+	+
16	Growth on pH				
	pH 5	+	+	+	+
	pH 9	+	+	+	+
17	Urease	-	-	-	-
18	Growth at Temperature				
	4°C	-	+	-	-
	37°C	+	+	+	+
	50°C	+	-	+	+
19	Acid production from				
	Glucose	+	-	+	+
	Maltose	+	-	-	-
	Fructose	+	-	-	+
	Sucrose	+	-	-	+
	Xylose	+	-	+	+
20	Growth on MacConky's agar	-	-	-	-
21	Growth on EMB agar	-	-	-	-
22	Bile salt tolerance	+	+	+	+

Table- 4.14 Result of Biochemical Tests for Samples - (Ku-1 to Ku-5)

Sr. No.	Name of the Test	Ku-1	Ku-2	Ku-3	Ku-4	Ku-5
1	Gram's stain	Variable	+	+	+	Variable
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	-	-	-	-	+
6	Casein hydrolysis	-	-	-	-	-
7	Gelatin hydrolysis	-	-	-	-	-
8	Indole production	-	-	-	-	-
9	Motility	+	+	+	-	-
10	Catalase	+	+	+	+	+
11	Phenyl-alanine deaminase	-	-	-	-	-
12	Nitrate reduction	+	+	+	+	-
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	+	+
	Maltose	-	-	-	-	+
	Fructose	-	+	+	+	+
	Sucrose	-	+	+	-	+
	Xylose	+	+	-	-	+
	Mannitol	-	-	-	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

Table- 4.15 Result of Biochemical Tests for Samples - (Ku-6 to Ku-10)

Sr. No.	Name of the Test	Ku-6	Ku-7	Ku-8	Ku-9	Ku-10
1	Gram's stain	+	+	Variable	+	+
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	-	-	+	-	-
6	Casein hydrolysis	-	-	+	+	+
7	Gelatin hydrolysis	-	-	+	+	+
8	Indole production	-	-	-	-	-
9	Motility	+	-	-	+	-
10	Catalase	+	+	+	+	+
11	Phenyl-alanine deaminase	-	-	-	-	-
12	Nitrate reduction	-	+	-	-	-
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	+	+
	Maltose	+	-	-	+	+
	Fructose	+	-	-	-	-
	Sucrose	+	-	-	-	-
	Xylose	+	-	+	+	+
	Mannitol	-	-	-	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

Table- 4.16 Result of Biochemical Tests for Samples - (Ku-11 to Ku-15)

Sr. No	Name of the Test	Ku-11	Ku-12	Ku-13	Ku-14	Ku-15
1	Gram's stain	+	+	+	Variable	Variable
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	-	+	-	+	-
6	Casein hydrolysis	-	+	-	+	-
7	Gelatin hydrolysis	-	+	-	+	-
8	Indole production	-	-	-	+	+
9	Motility	+	+	+	+	+
10	Catalase	+	+	+	+	+
11	Phenyl- alanine deaminas	-	-	-	-	-
12	Nitrate reduction	+	+	+	+	+
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	-	+
	Maltose	-	+	+	-	-
	Fructose	-	-	+	-	-
	Sucrose	-	-	-	-	-
	Xylose	-	+	+	-	-
	Mannitol	-	-	-	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

Table- 4.17 Result of Biochemical Tests for Samples - (Ku-16 to Ku-20)

Sr. No	Name of the Test	Ku-16	Ku-17	Ku-18	Ku-19	Ku-20
1	Gram's stain	+	Variable	Variable	Variable	+
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	+	-	-	-	+
6	Casein hydrolysis	+	+	+	-	+
7	Gelatin hydrolysis	+	+	+	-	+
8	Indole production	-	-	-	-	-
9	Motility	+	-	-	-	-
10	Catalase	+	+	+	+	+
11	Phenyl- alanine deaminas	-	-	-	-	-
12	Nitrate reduction	+	+	+	+	+
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	+	+
	Maltose	-	+	+	+	-
	Fructose	+	+	+	-	-
	Sucrose	+	+	+	+	-
	Xylose	-	-	-	+	+
	Mannitol	-	-	-	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

Table- 4.18 Result of Biochemical Tests for Samples - (Ku-21 to Ku-25)

Sr. No	Name of the Test	Ku-21	Ku-22	Ku-23	Ku-24	Ku-25
1	Gram's stain	+	Variable	+	+	Variable
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	-	-	-	-	-
6	Casein hydrolysis	+	-	-	-	-
7	Gelatin hydrolysis	+	-	-	-	-
8	Indole production	-	-	-	+	+
9	Motility	-	+	+	+	+
10	Catalase	+	+	+	+	+
11	Phenyl- alanine deaminas	-	-	-	-	-
12	Nitrate reduction	+	+	+	+	+
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	+	+
	Maltose	+	+	-	+	+
	Fructose	+	+	-	+	+
	Sucrose	+	-	-	-	-
	Xylose	+	+	-	+	+
	Mannitol	-	-	-	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

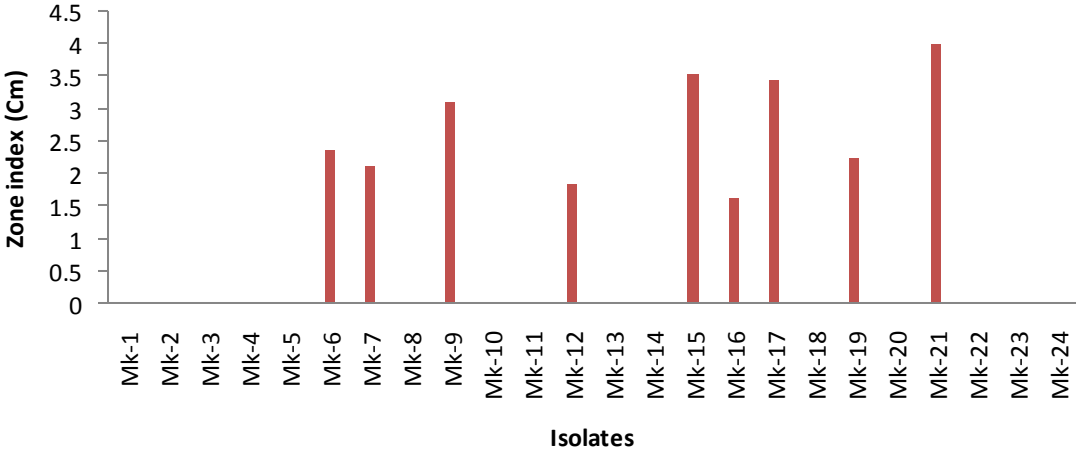
Table- 4.19 Result of Biochemical Tests for Samples - (Ku-26 to Ku-30)

Sr. No	Name of the Test	Ku-26	Ku-27	Ku-28	Ku-29	Ku-30
1	Gram's stain	+	Variable	+	Variable	Variable
2	Capsule Stain	-	-	-	-	-
3	Cell shape					
4	H_2S production	-	-	-	-	-
5	Starch hydrolysis	-	+	-	-	-
6	Casein hydrolysis	-	+	-	-	+
7	Gelatin hydrolysis	-	+	-	-	+
8	Indole production	-	+	-	-	+
9	Motility	+	-	+	+	+
10	Catalase	+	+	+	+	+
11	Phenyl- alanine deaminas	-	-	-	-	-
12	Nitrate reduction	-	-	-	+	-
13	Lipolytic	+	+	+	+	+
14	Urease	-	-	-	-	-
15	Acid production from					
	Glucose	+	+	+	-	+
	Maltose	+	-	-	-	-
	Fructose	+	+	+	+	+
	Sucrose	+	-	-	-	-
	Xylose	+	+	+	-	+
	Mannitol	-	-	+	-	-
16	Growth on MacConky's agar	-	-	-	-	-
17	Growth on EMB agar	-	-	-	-	-
18	Bile salt tolerance	-	-	-	-	-

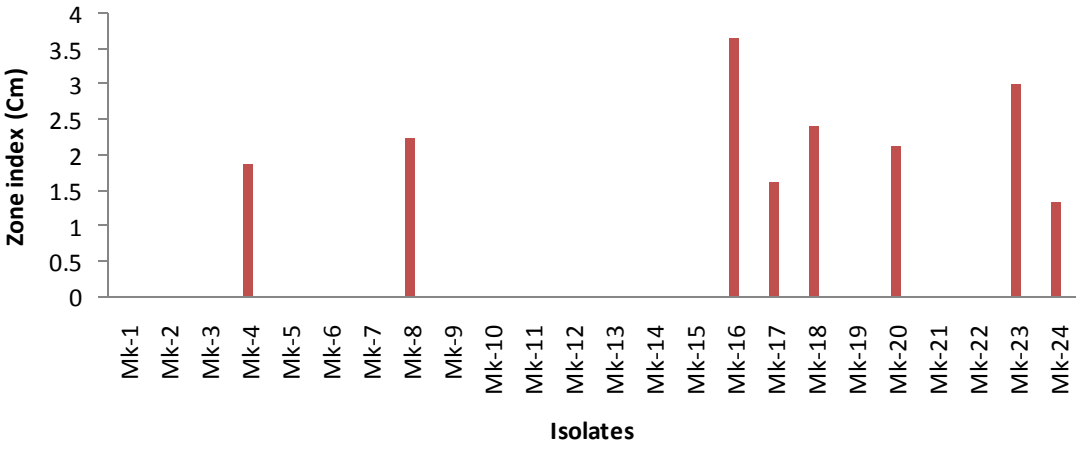
- ❖ Moderate halophiles isolated from wild ass excreta were highly diversified in terms of extracellular hydrolytic enzyme production viz. lipase, protease, amylase, cellulase and chitinase (Graph- 4.1 to 4.5).
- ❖ All the extremophiles have the ability to secrete extracellular lipase while none of them were able to produce extracellular chitinases or cellulases (Graph- 4.6 to 4.8).

Enyme production profile from moderate halophiles

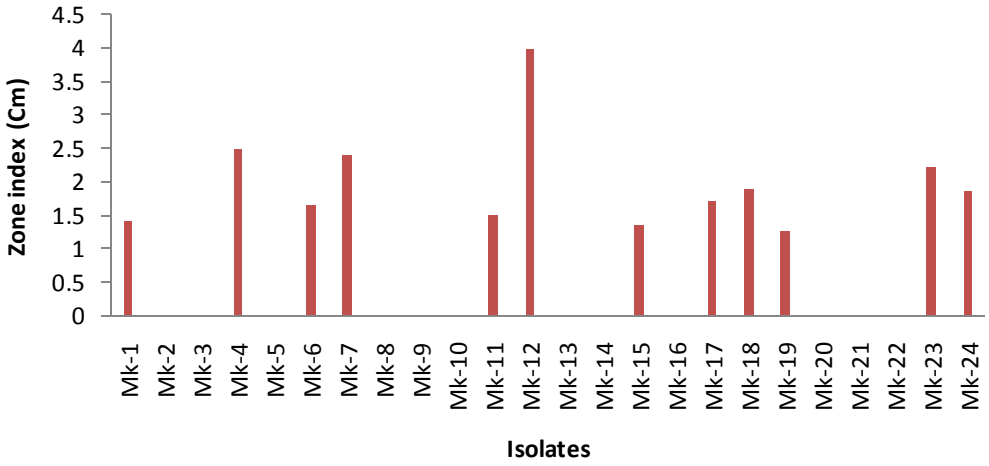
Graph- 4.1 Amylase Production



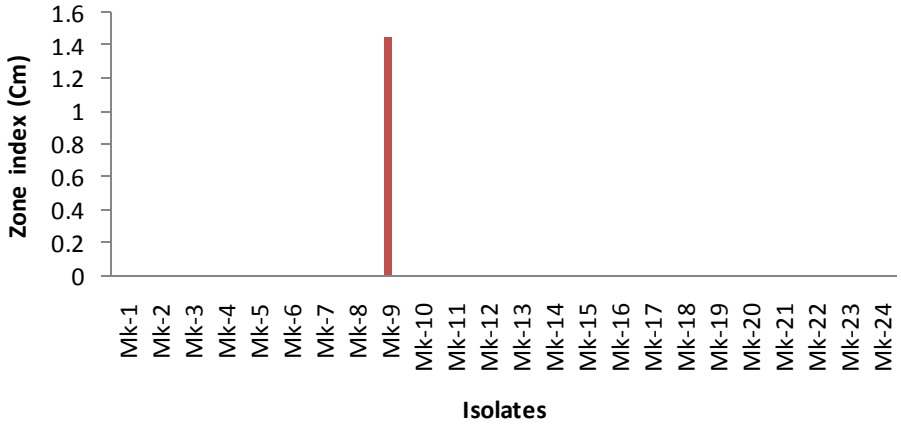
Graph- 4.2 Lipase Production



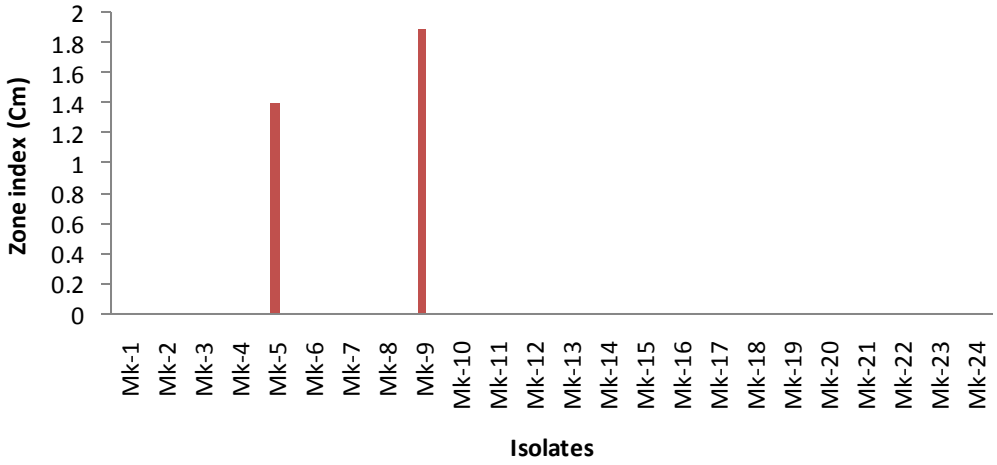
Graph- 4.3 Protease Production



Graph- 4.4 Cellulase Production

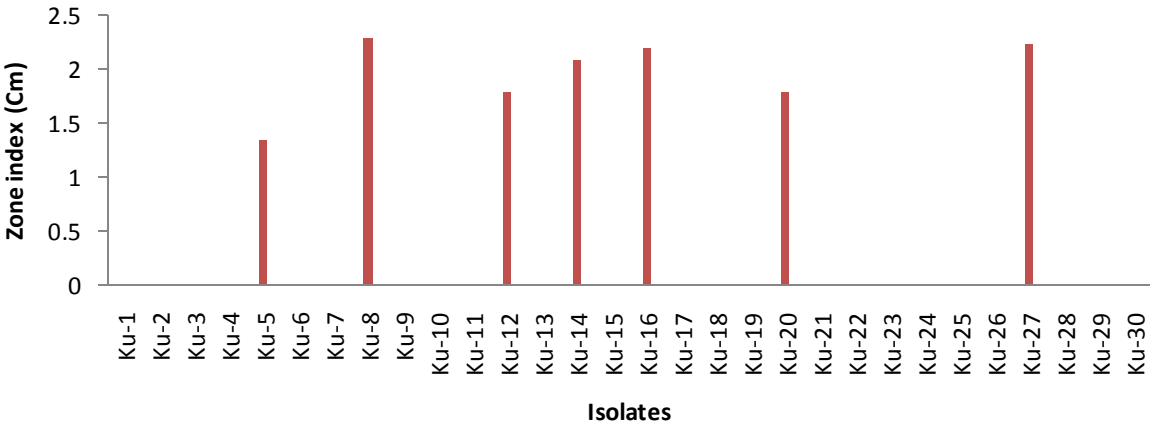


Graph- 4.5 Chitinase Production

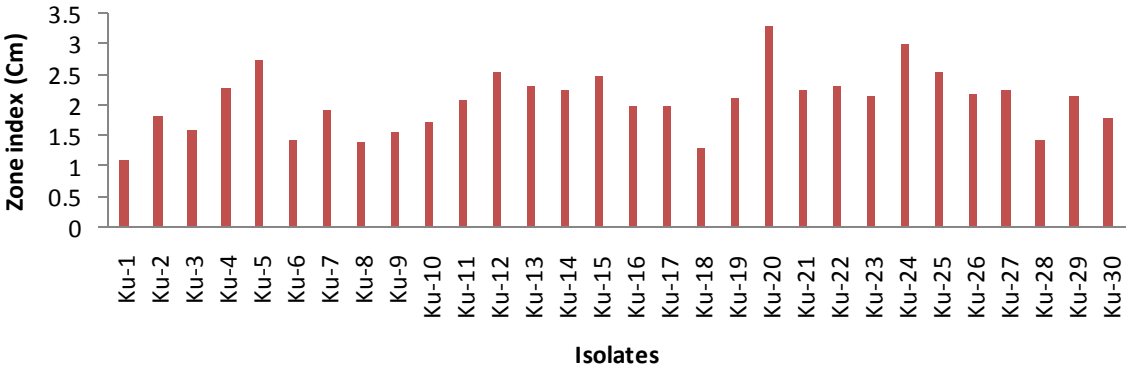


Enyme production profile from extreme halophiles

Graph- 4.6 Amylase Production



Graph- 4.7 Lipase Production

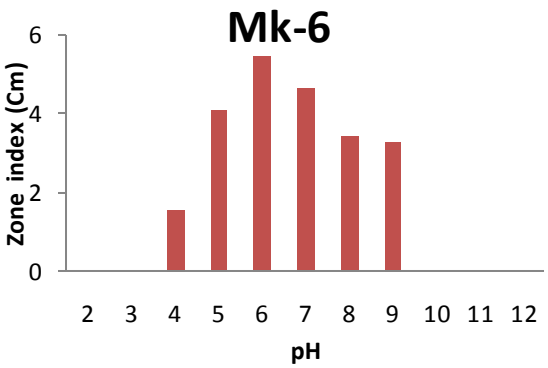


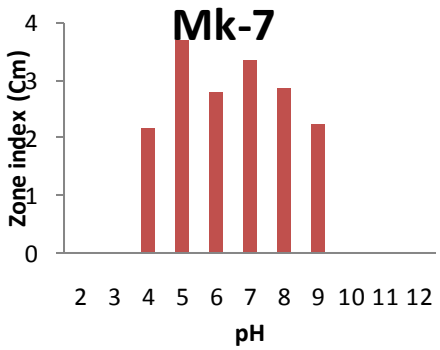
Graph- 4.8 Protease

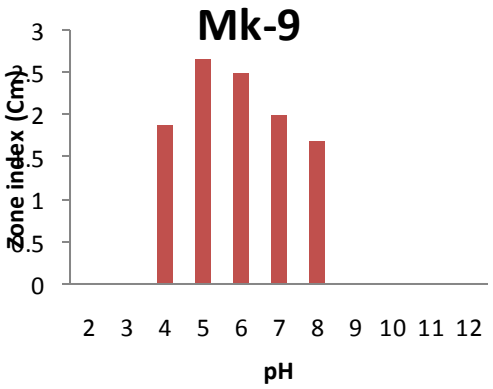
Parameter optimization for amylase production by moderate halophiles on solid media

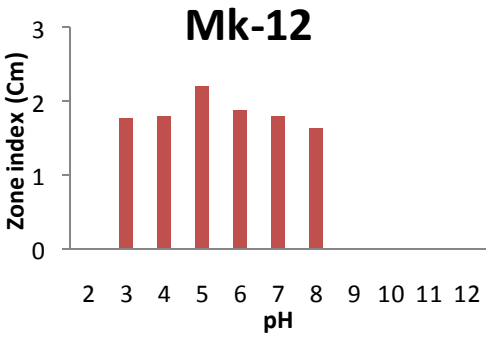
- ❖ pH was found to have significant effect on enzyme production profile of all the isolates effect of pH was studied for the production of Lipase and Amylase enzymes only as these two enzymes have been found to be produced extensively by halophiles studied.

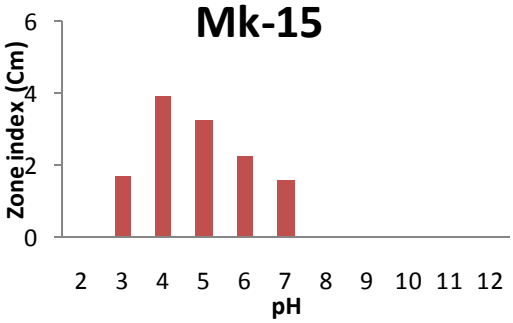
Graphs 4.9 Effect of pH

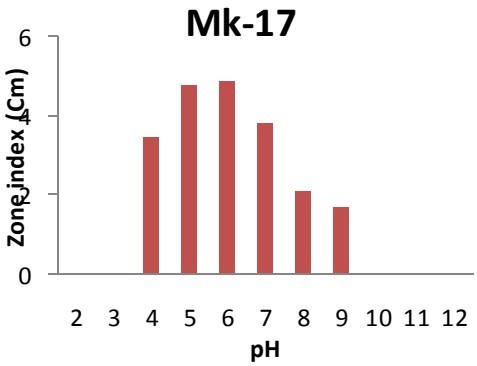


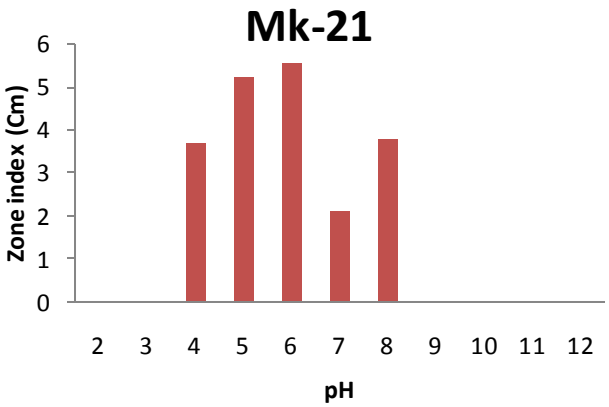






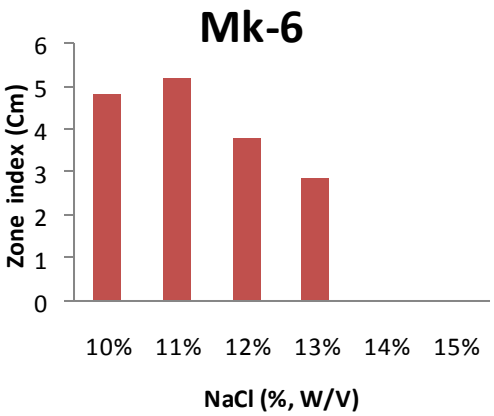


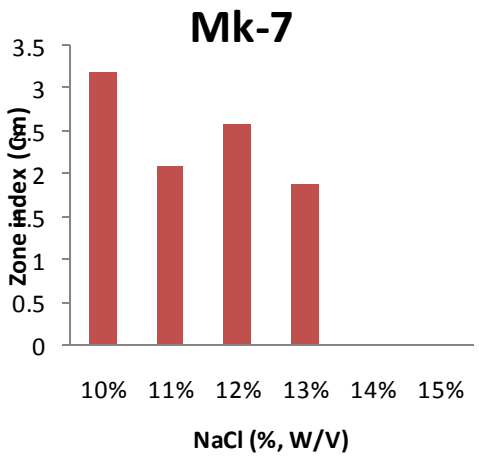


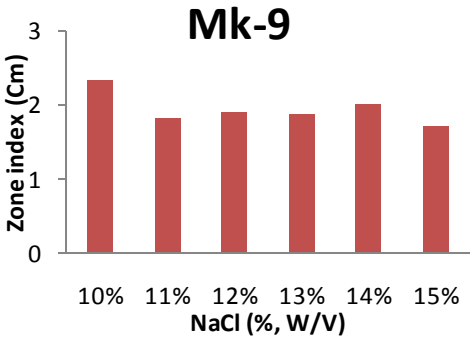


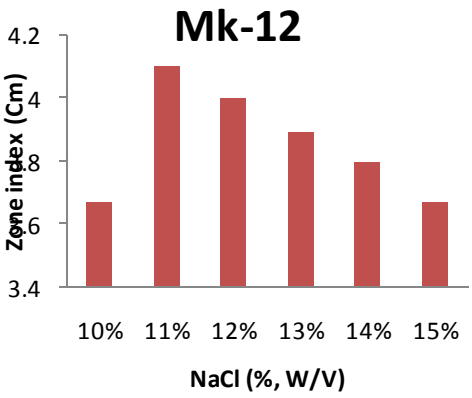
❖ All the moderate halophiles could grow well up to 13% NaCl (W/V) but some isolate failed to grow above 13% NaCl. Highest amylase secretion was at 10% and 11% NaCl concentrations. Increase in salt concentration lead to decreased amylase production (Graphs- 4.10).

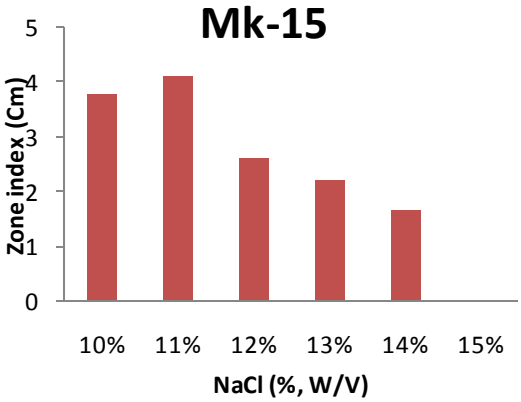
Graphs 4.10 Effect of NaCl

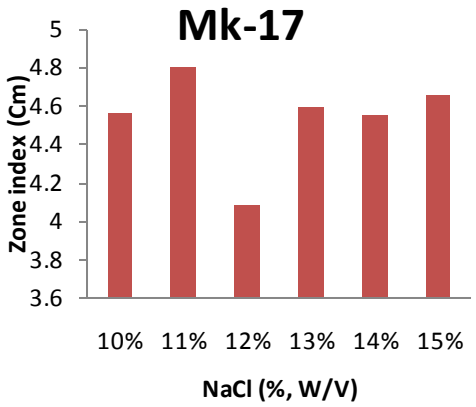


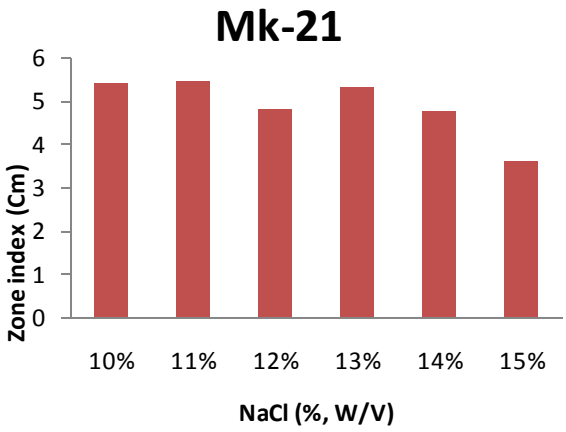






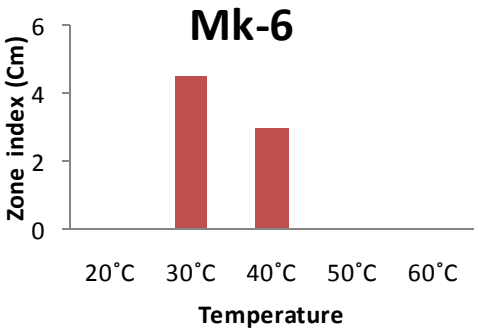


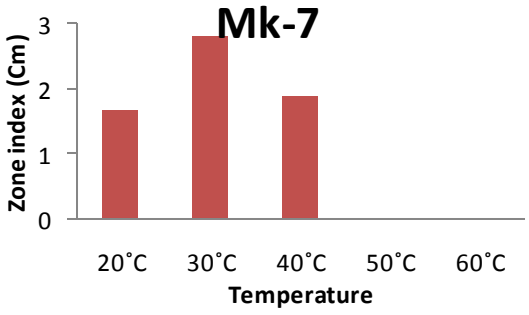


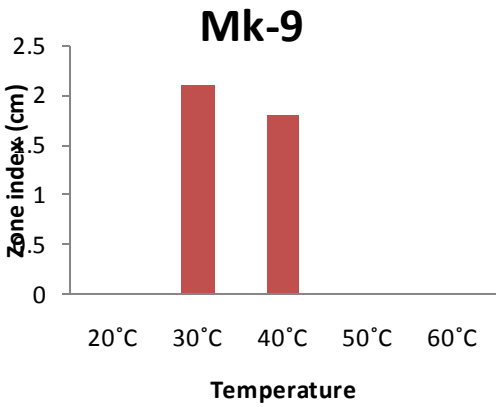


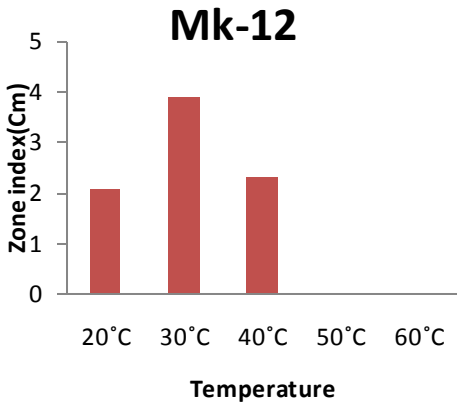
❖ All the moderate halophiles preferred to grow and produce highest amount of amylase at 30˚C. Lower or higher temperature affected growth and enzyme secretion (Graphs- 4.11).

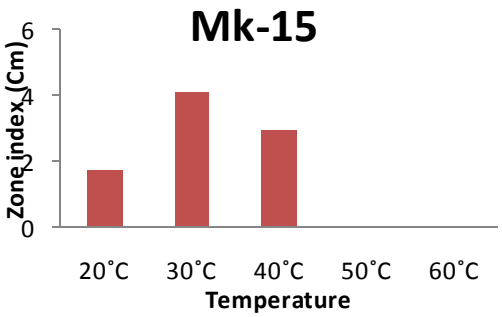
Graph 4.11 Effect of Temperature

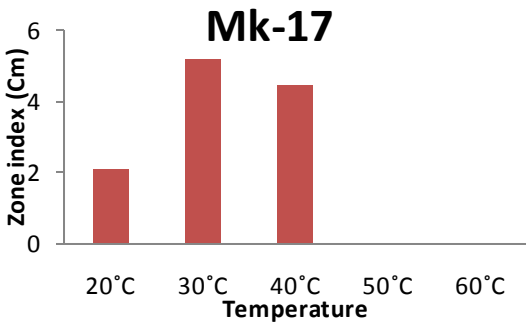





Mk-9
Zone index (cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Mk-12
Zone index(Cm)
5
4
3
2
1
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Mk-15
Zone index (Cm)
6
4
2
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Mk-17
Zone index (Cm)
6
4
2
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

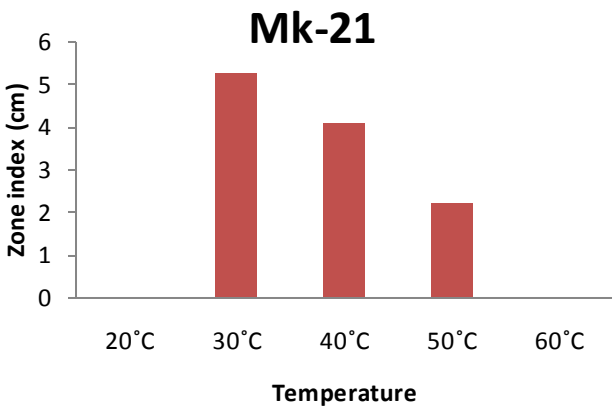

Mk-21
Zone index (cm)
6
5
4
3
2
1
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

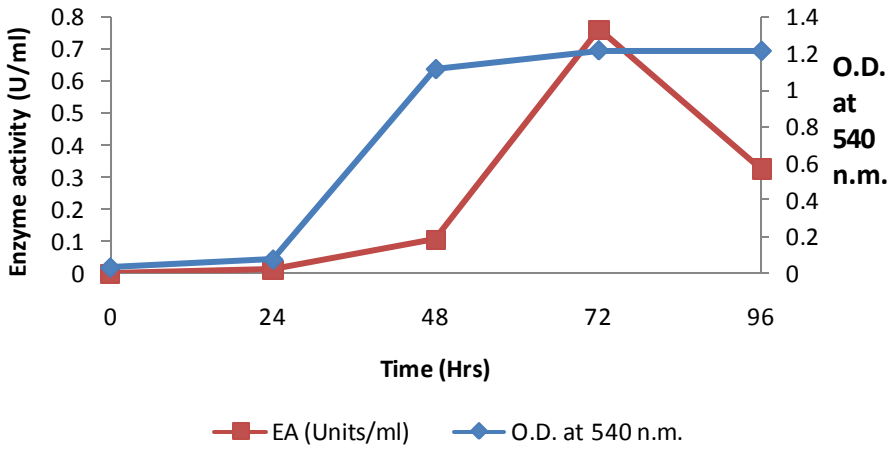
Table-4.20 Optimum and range of pH, NaCl and temperature for amylase production from moderate halophiles

Sr. No	Isolates	pH		NaCl (%)		Temperature (˚C)	
		Optimum	Range	Optimum	Range	Optimum	Range
1	Mk-6	6	4-9	11	10-13	30	30-40
2	Mk-7	5	4-9	10	10-13	30	20-40
3	Mk-9	5	4-8	10	10-15	30	30-40
4	Mk-12	5	3-8	11	10-15	30	20-40
5	Mk-15	4	3-7	11	10-14	30	20-40
6	Mk-17	6	4-9	11	10-15	30	20-40
7	Mk-21	6	4-8	10,11,13	10-15	30	30-50

Parameter optimization for amylase production by moderate halophiles on liquid media

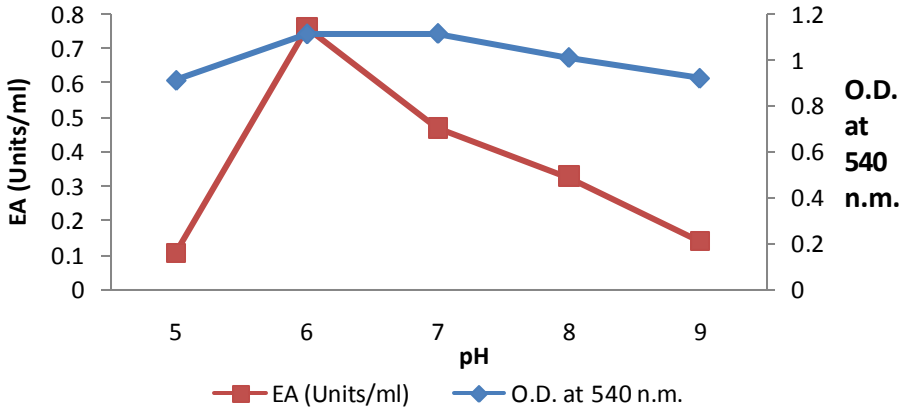
- Mk-21 was found to be best amylase producer. It passed through lag phage in initial 24 hours and then entered into log phase (Graph- 4.12).

Graph 4.12 Growth and amylase production pattern of Mk-21



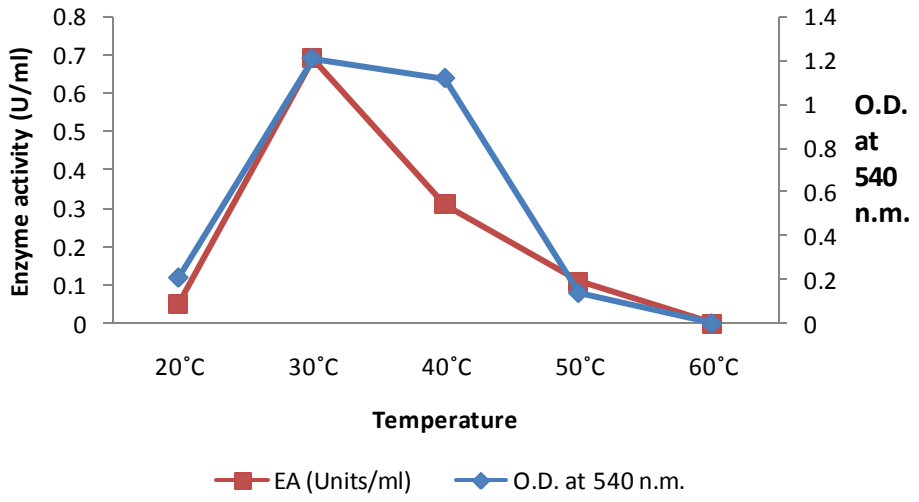
❖ Mk-21 produced maximum amylase and biomass at pH 6.0 (Graphs- 4.13).

Graph 4.13 Effect of pH



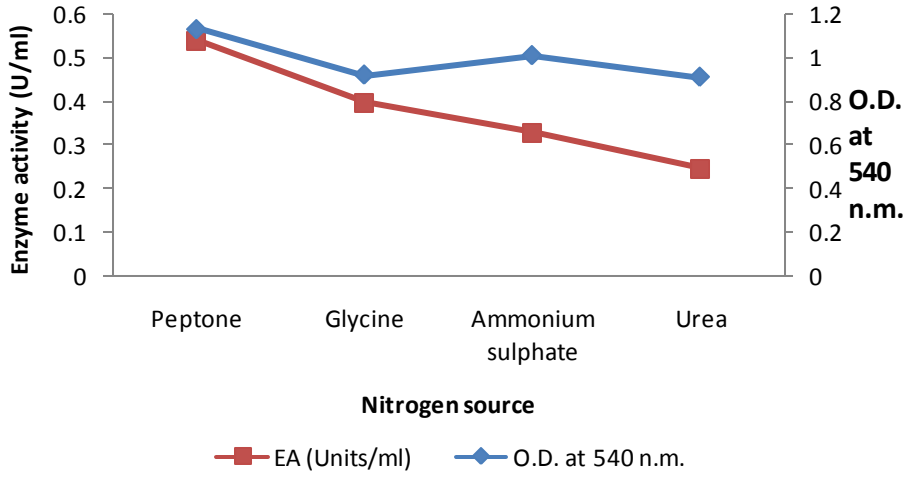
❖ Mk-21 was able to produce maximum amylase as well as biomass at 30˚C (Graph-4.14).

Graph 4.14 Effect of temperature on biomass and amylase production from Mk-21



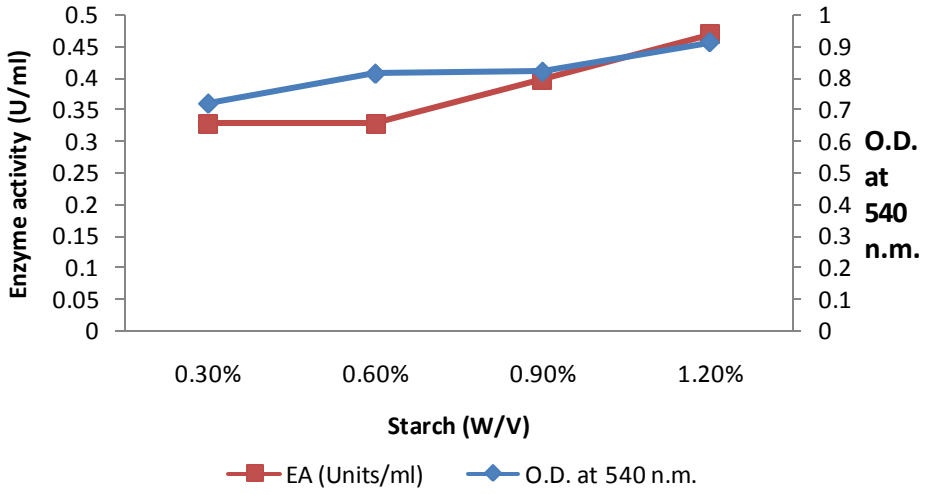
❖ Among various nitrogen sources like peptone, glycine, ammonium sulphate and urea, best nitrogen source for maximum biomass and enzyme production was found to be peptone (Graph- 4.15).

Graph 4.15 Effect of nitrogen sources



❖ 1.2% starch (W/V) was found to be the best for enzyme and biomass production (Graph- 4.16).

Graph 4.16 Effect of starch

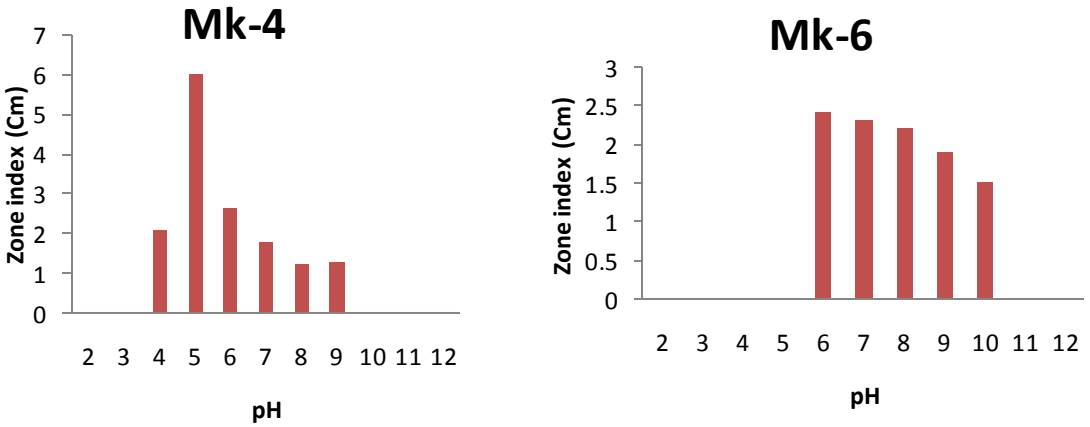


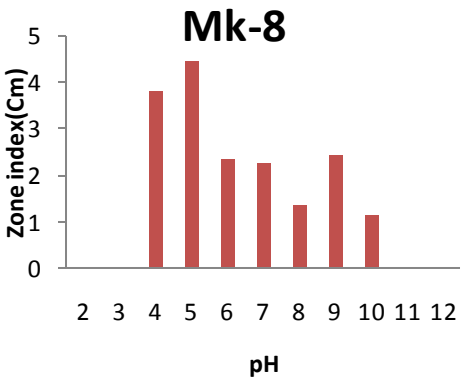
❖ On the basis of substrate curve, Km and Vmax were found to be 1 mg/ml and 1 µm/min respectively.

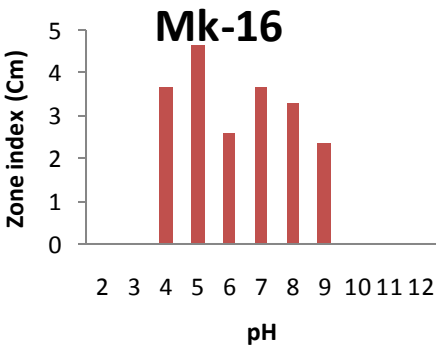
Effect of pH, NaCl, Temperature and Substrate concetration on lipase production by moderate halophiles

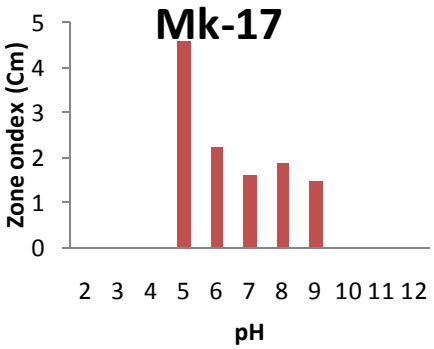
❖ Acidic to neutral pH was found suitable for lipase production on solid media (Graphs- 4.17).

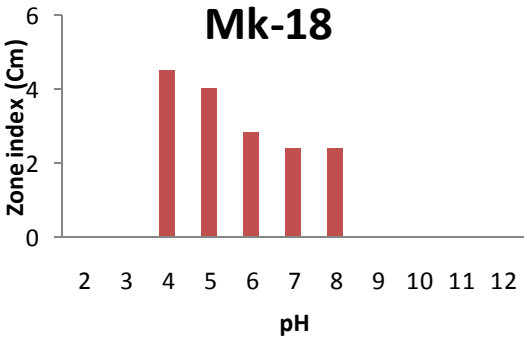
Graphs- 4.17 Effect of pH

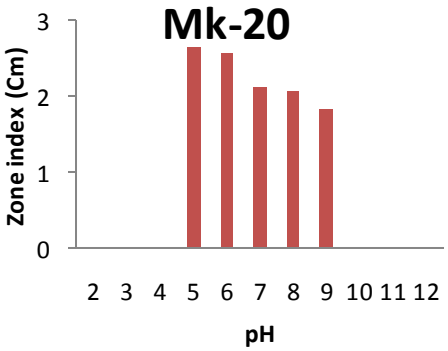


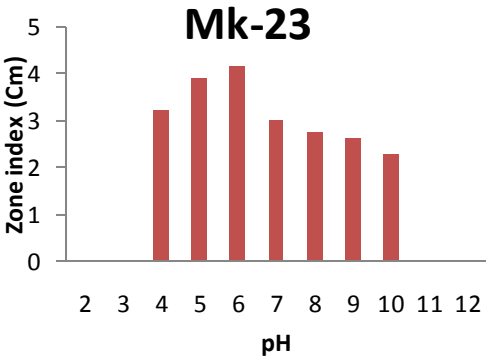

Mk-8
Zone index(Cm)
0 1 2 3 4 5
2 3 4 5 6 7 8 9 10 11 12
pH

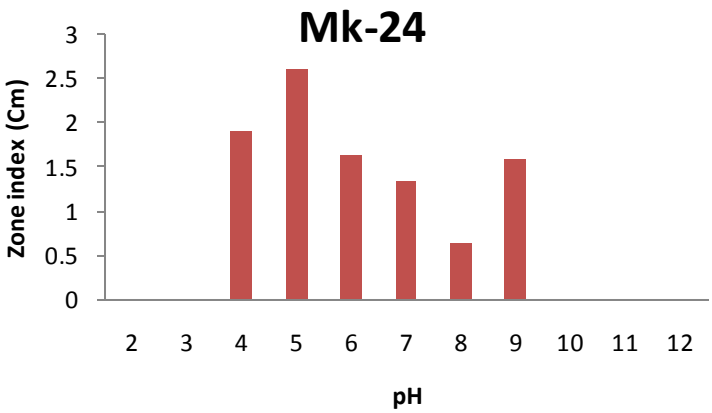

Mk-16
Zone index (Cm)
0 1 2 3 4 5
2 3 4 5 6 7 8 9 10 11 12
pH


Mk-17
Zone ondex (Cm)
0 1 2 3 4 5
2 3 4 5 6 7 8 9 10 11 12
pH


Mk-18
Zone index (Cm)
0 2 4 6
2 3 4 5 6 7 8 9 10 11 12
pH

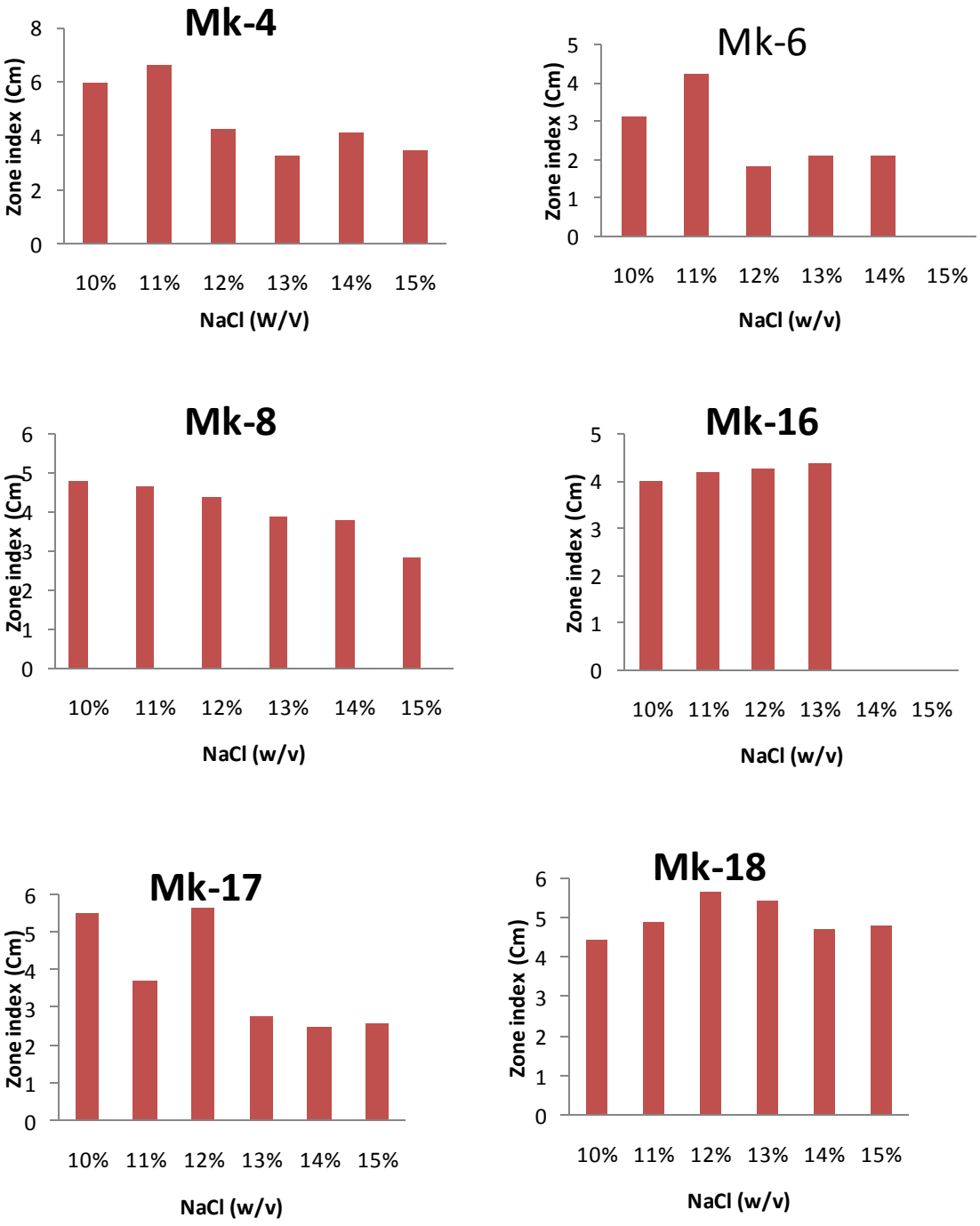

Mk-20
Zone index (Cm)
0 1 2 3
2 3 4 5 6 7 8 9 10 11 12
pH

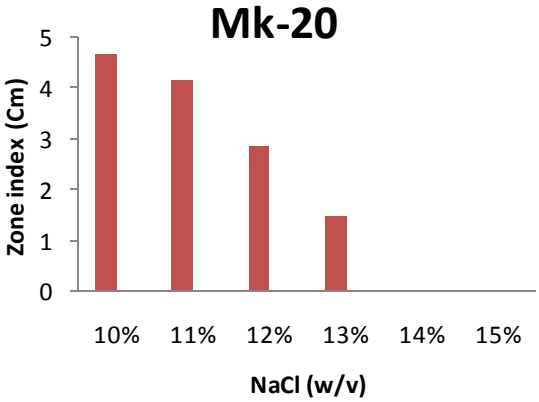

Mk-23
Zone index (Cm)
0 1 2 3 4 5
2 3 4 5 6 7 8 9 10 11 12
pH

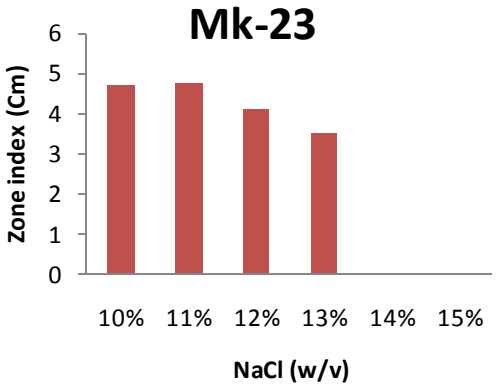

Mk-24
Zone index (Cm)
0 0.5 1 1.5 2 2.5 3
2 3 4 5 6 7 8 9 10 11 12
pH

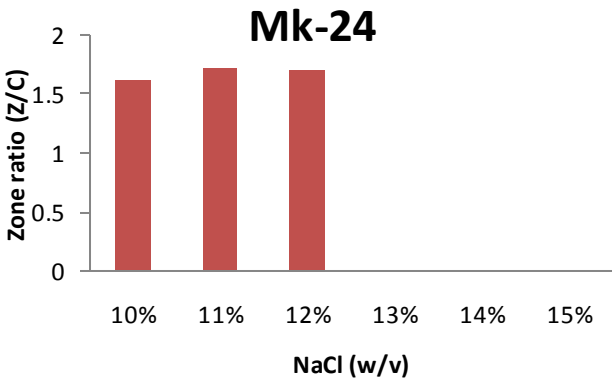
❖ Moderate halophiles were able to secrete maximum lipase on tributyrene agar media at salt concentration 10%-11% (Graphs- 4.18).

Graphs- 4.18 Effect of NaCl



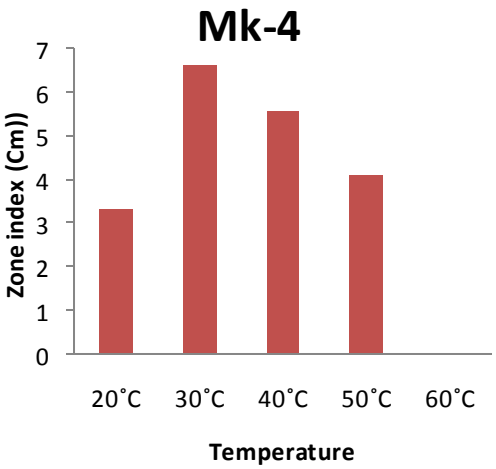


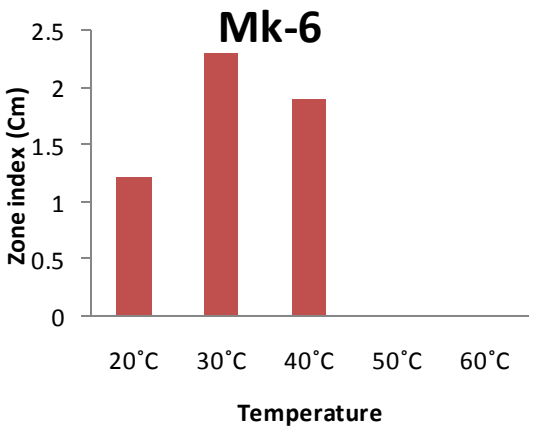


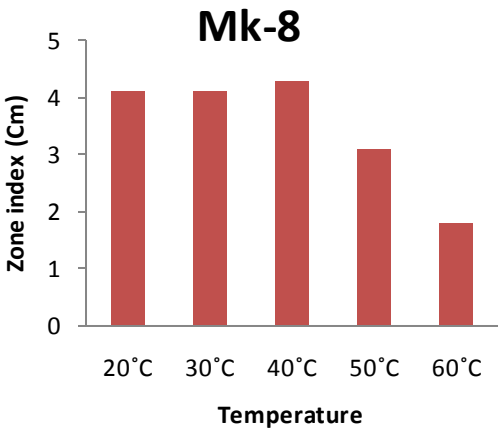


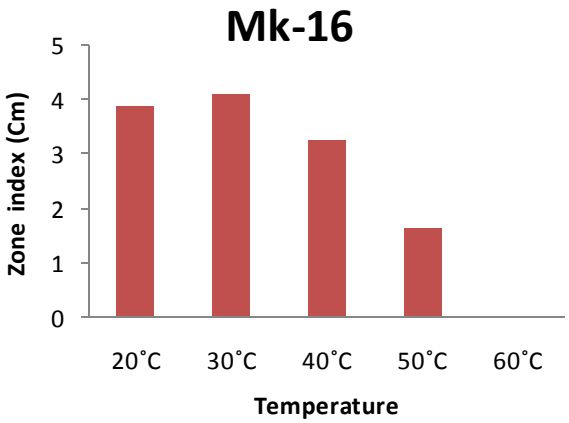
❖ Moderate halophiles prefer to grow and produce highest lipase at the temperature of 30˚C and 40˚C. Very few isolates could grow at 60˚C (Graphs- 4.19).

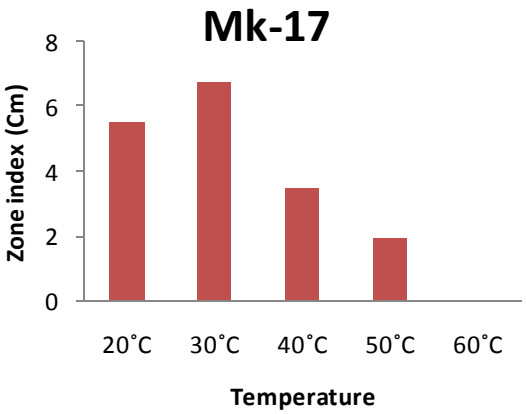
Graphs- 4.19 Effect of Temperature

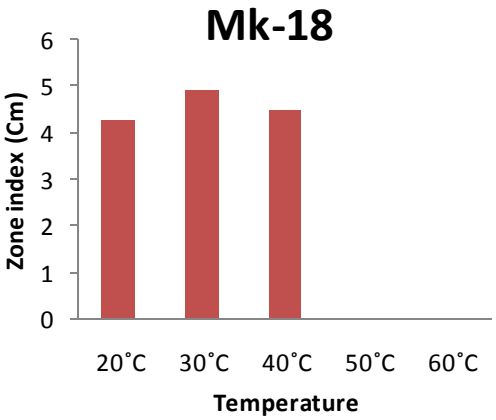


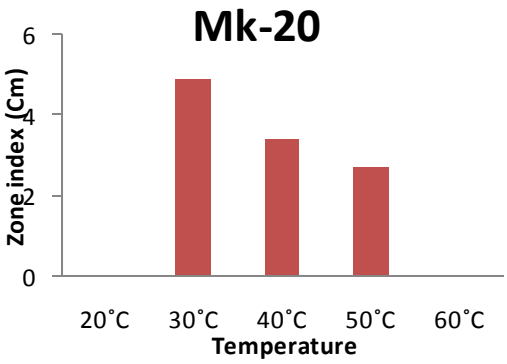


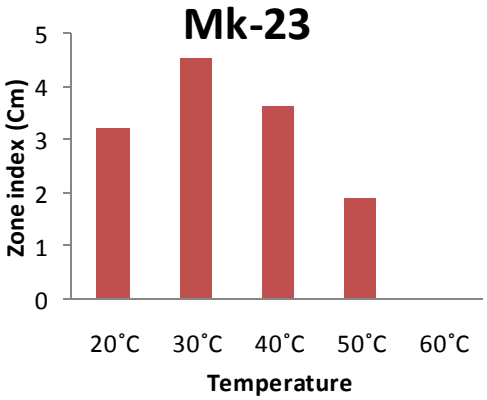

Mk-8
Zone index (Cm)
0
1
2
3
4
5
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

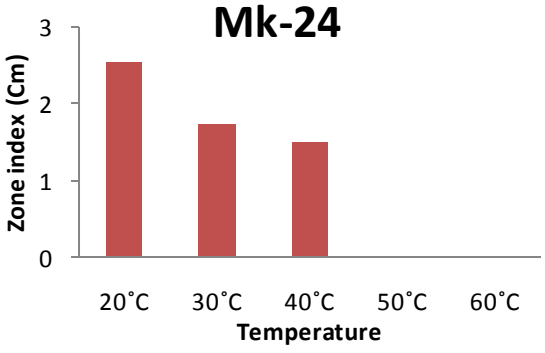

Mk-16
Zone index (Cm)
0
1
2
3
4
5
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Mk-17
Zone index (Cm)
0
2
4
6
8
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Mk-18
Zone index (Cm)
0
1
2
3
4
5
6
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

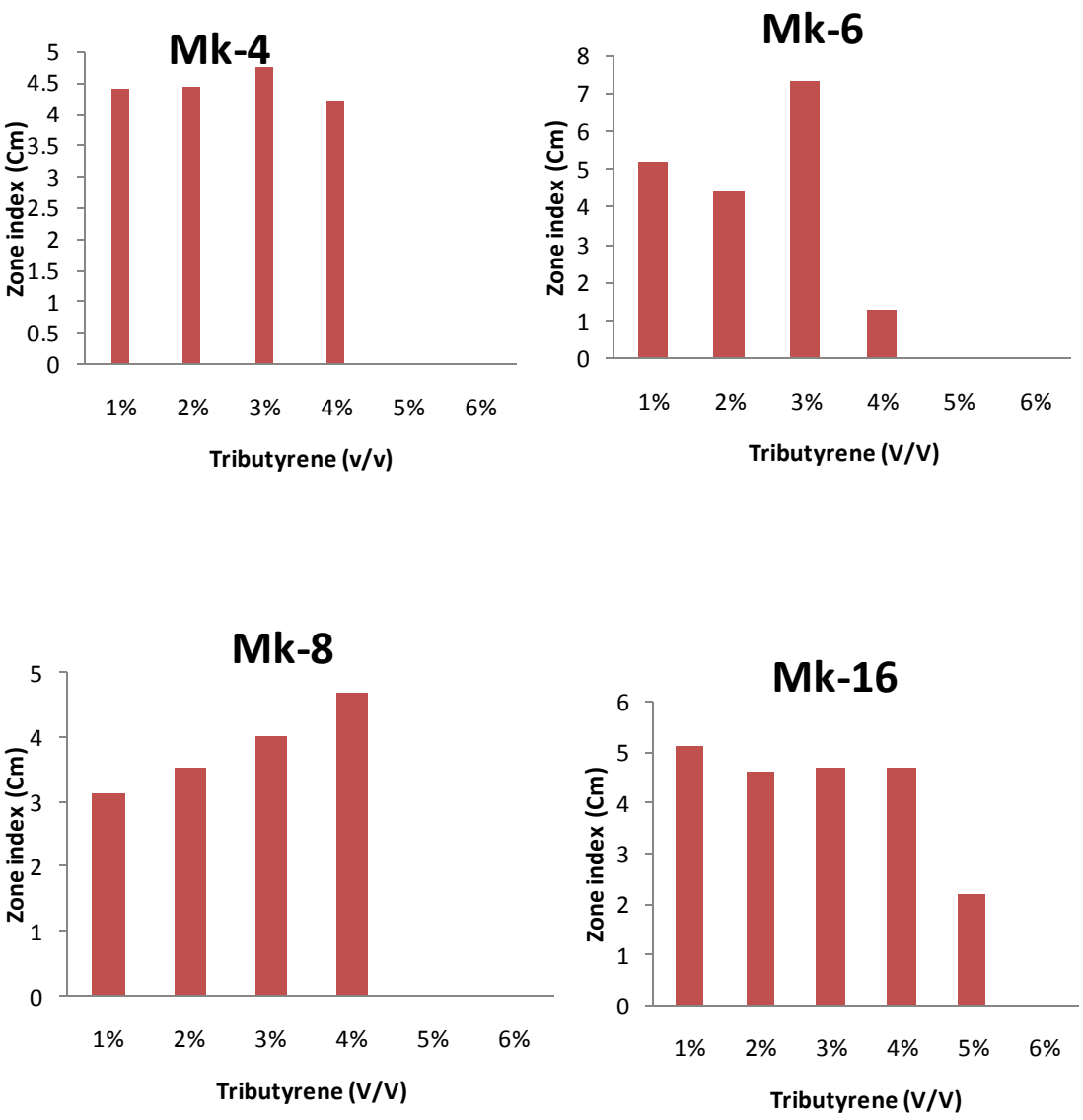

Mk-20
Zone index (Cm)
0
2
4
6
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

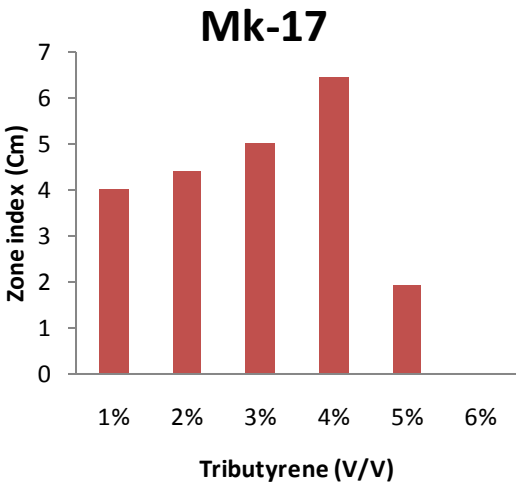

Mk-23
Zone index (Cm)
0
1
2
3
4
5
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

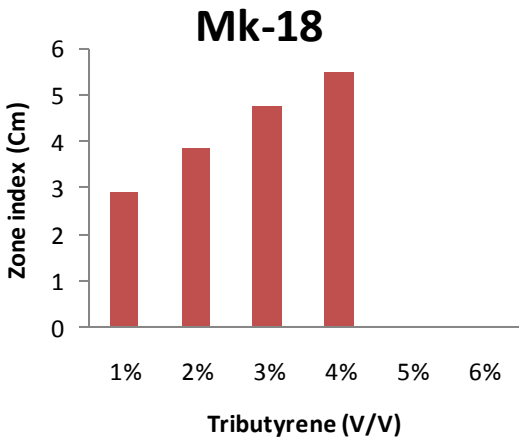

Mk-24
Zone index (Cm)
0
1
2
3
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

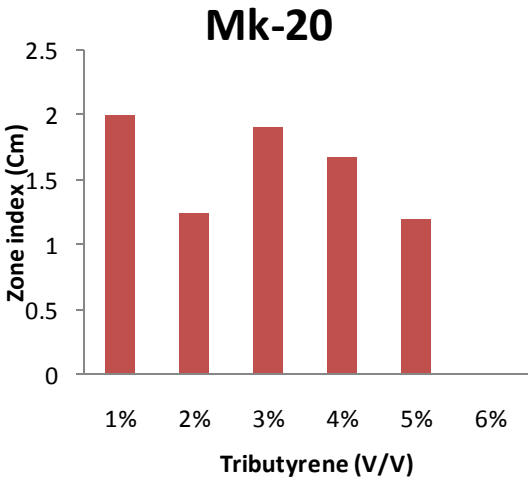
❖ Lipase secretion increased when tributyrene concentration increased from 1% to 4%. Tributyrene concentration above 5% adversely affected lipase production and even inhibited growth of most isolates (Graphs- 4.20).

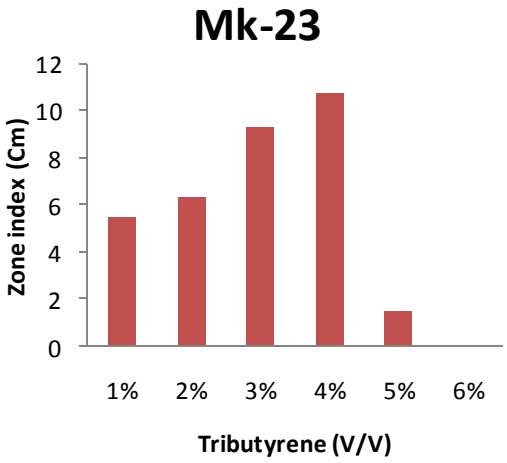
Graphs- 4.20 Effect of tributyrene concentration




Mk-17
Zone index (Cm)
0
1
2
3
4
5
6
7
1%
2%
3%
4%
5%
6%
Tributyrene (V/V)


Mk-18
Zone index (Cm)
0
1
2
3
4
5
6
1%
2%
3%
4%
5%
6%
Tributyrene (V/V)


Mk-20
Zone index (Cm)
0
0.5
1
1.5
2
2.5
1%
2%
3%
4%
5%
6%
Tributyrene (V/V)


Mk-23
Zone index (Cm)
0
2
4
6
8
10
12
1%
2%
3%
4%
5%
6%
Tributyrene (V/V)

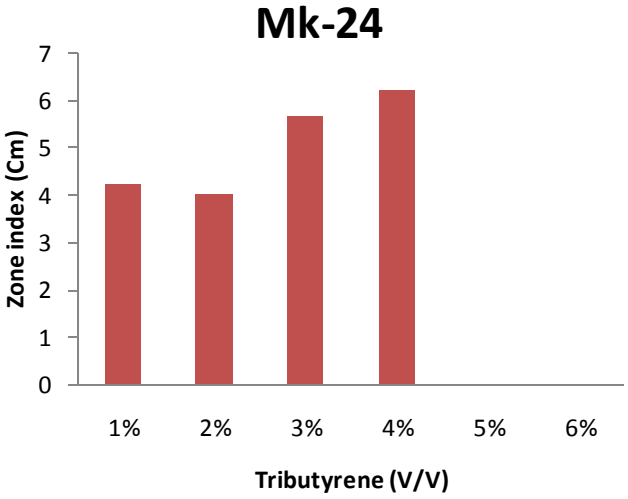

Mk-24
Zone index (Cm)
0
1
2
3
4
5
6
7
1%
2%
3%
4%
5%
6%
Tributyrene (V/V)

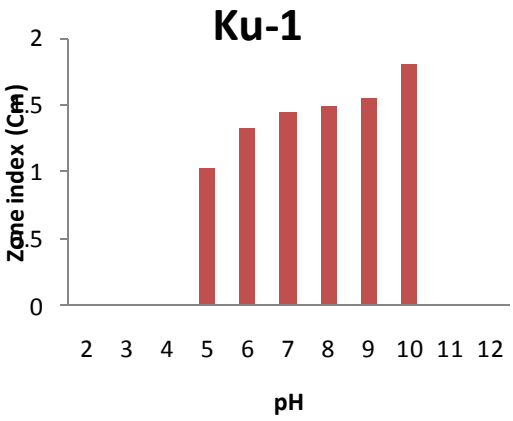
Table-4.21 Range and optimum pH, salt, temperature and Tributyrene requirement for lipase production from moderate halophiles.

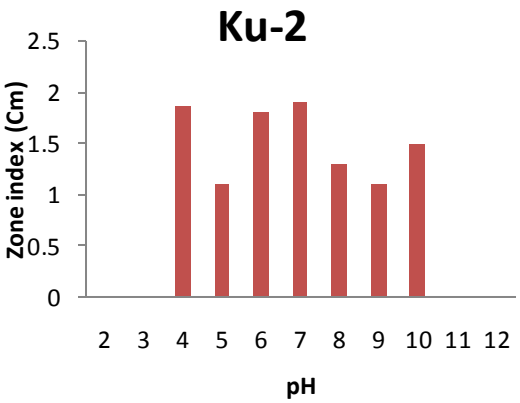
Sr .No	Isolates	pH		Salt (%)		Temperature (˚C)		Tributyrene (%)	
		Optimum	Range	Optimur	Range	Optimum	Range	Optimum	Range
1	Mk-4	5	4-9	11	10-15	30	20-50	3	1-4
2	Mk-6	6	6-10	11	10-14	30	20-40	3	1-4
3	Mk-8	5	4-10	10	10-15	30	20-60	4	1-4
4	Mk-16	5	4-9	13	10-13	40	20-50	1	1-5
5	Mk-17	5	5-9	12	10-15	30	20-50	4	1-5
6	Mk-18	4	4-8	12	10-15	30	20-40	4	1-4
7	Mk-20	5	5-9	10	10-13	30	30-50	1	1-5
8	Mk-23	6	4-10	11	10-13	30	20-50	4	1-5
9	Mk-24	5	4-9	11-12	10-12	20	20-40	4	1-5

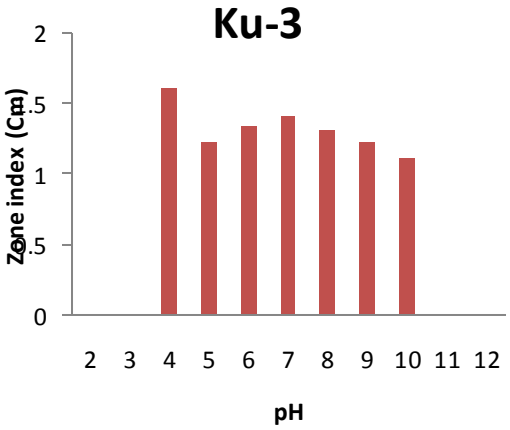
Effect of pH, NaCl, Temperature and Substrate concetration on lipase production by extreme halophiles

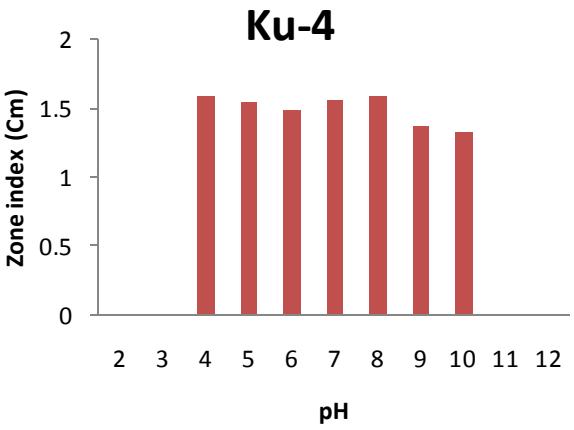
- None of the organisms among 30 extreme halophiles could grow at acidic pH 2 and 3 as well as alkaline pH 11 and 12. Maximum lipase production was achieved at neutral pH (pH 7 and 8) in case of majority of isolates (Graphs-4.21).

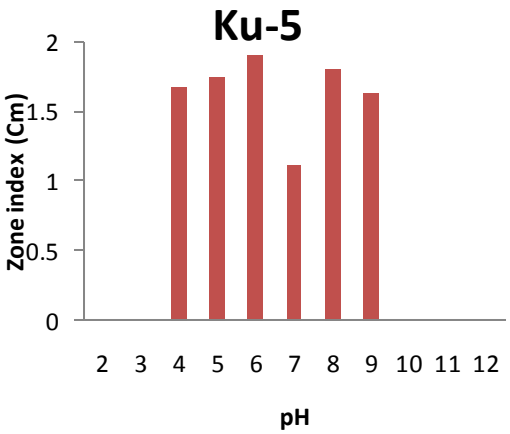
Graphs- 4.21Effect of pH

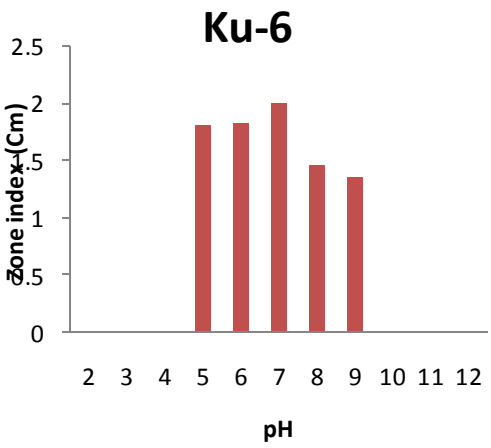


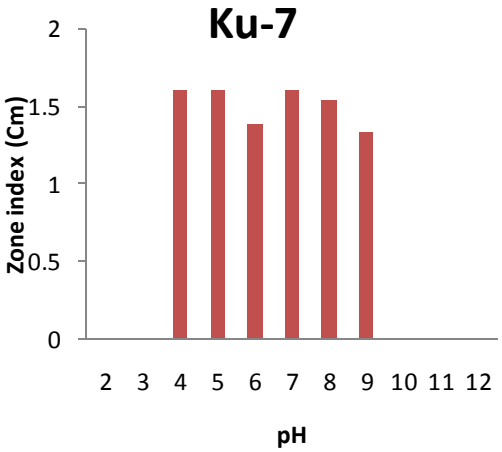


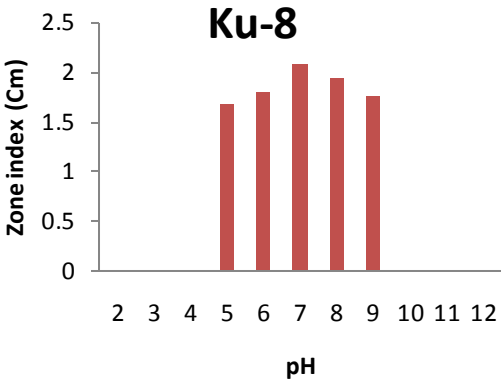


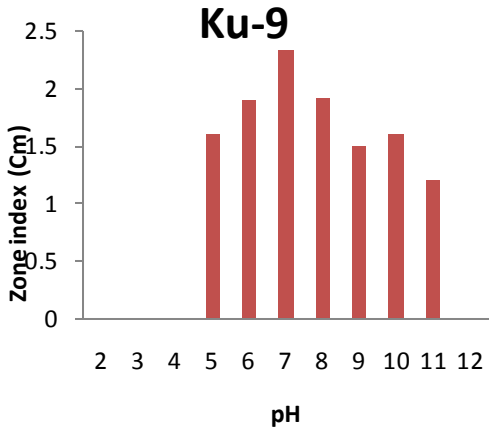


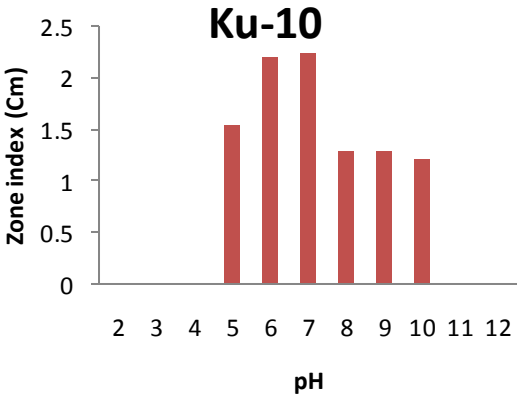


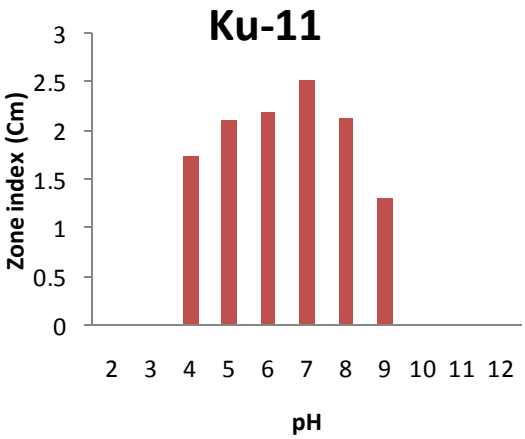


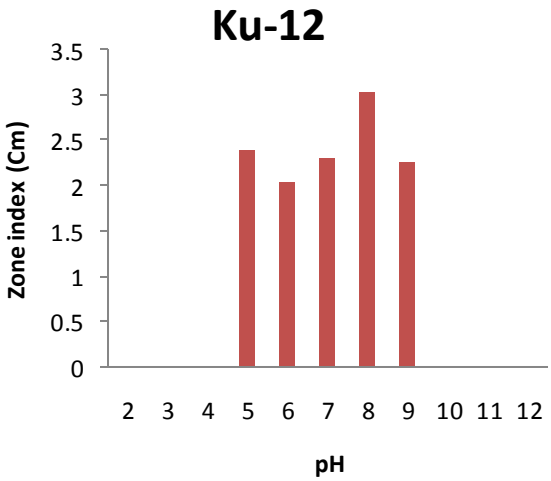

Ku-7
Zone index (Cm)
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

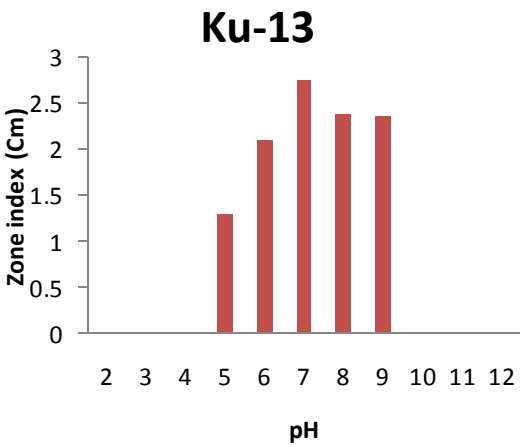

Ku-8
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

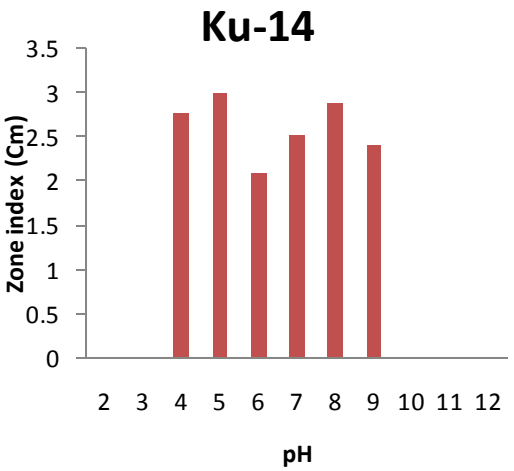

Ku-9
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

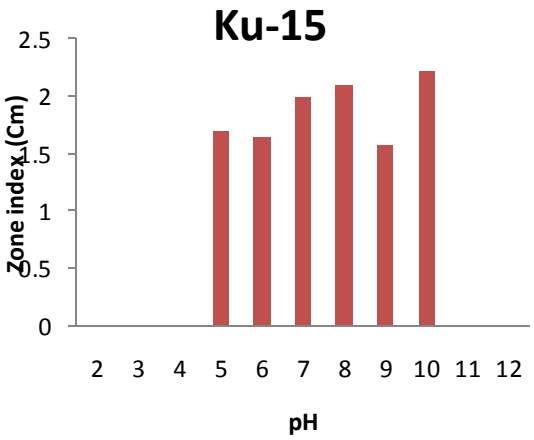

Ku-10
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

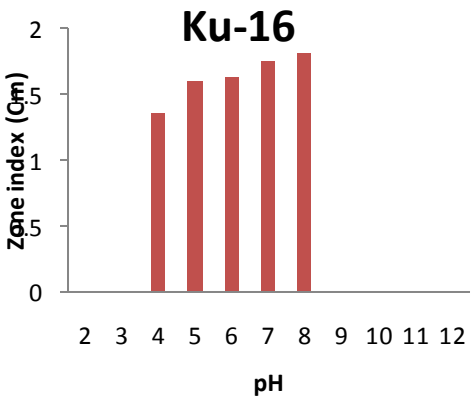

Ku-11
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

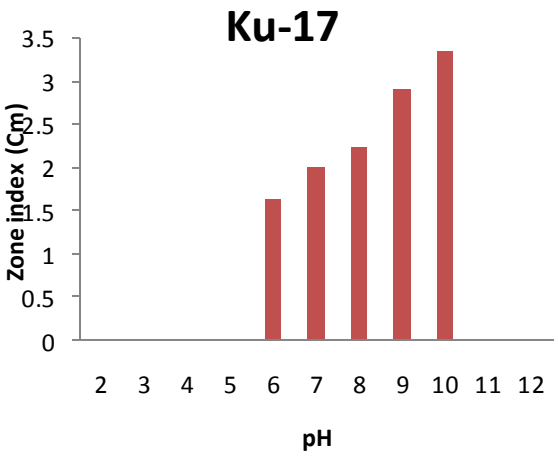

Ku-12
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

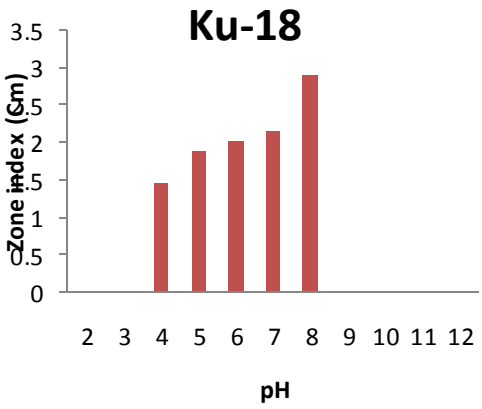

Ku-13
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

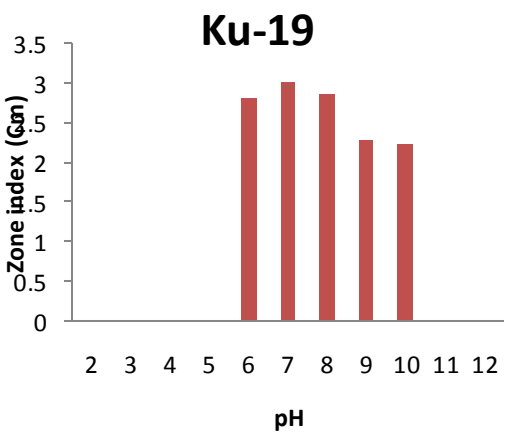

Ku-14
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

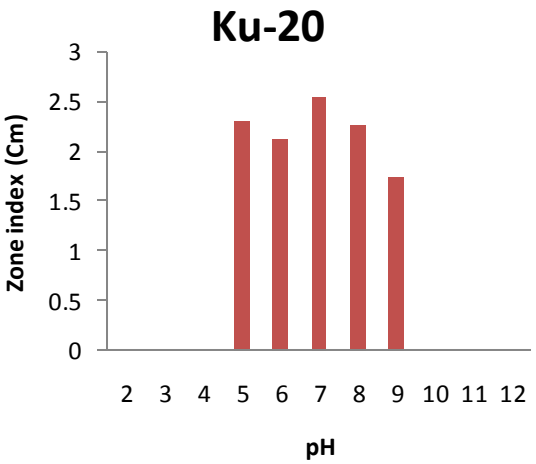

Ku-15
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

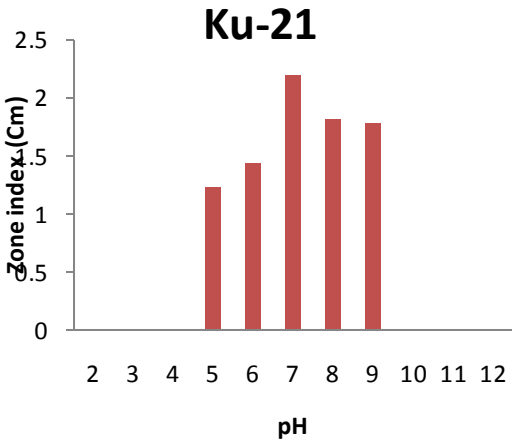

Ku-16
Zone index (Cm)
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

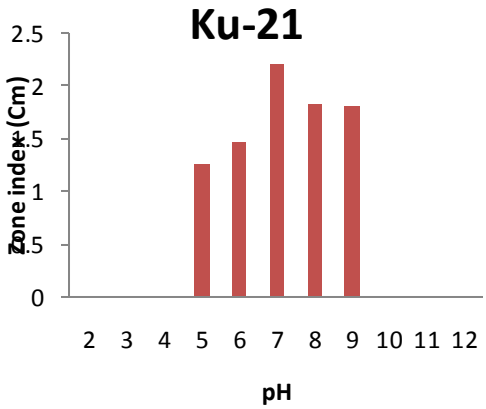

Ku-17
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

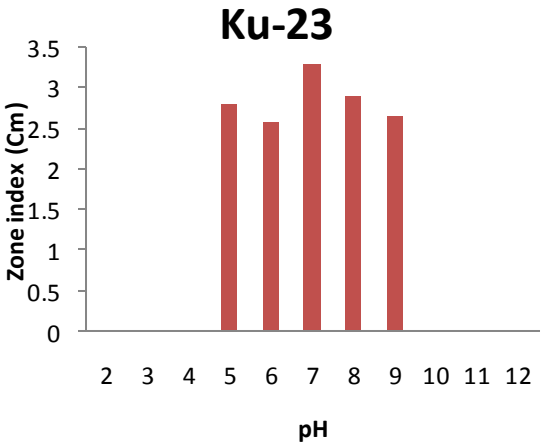

Ku-18
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

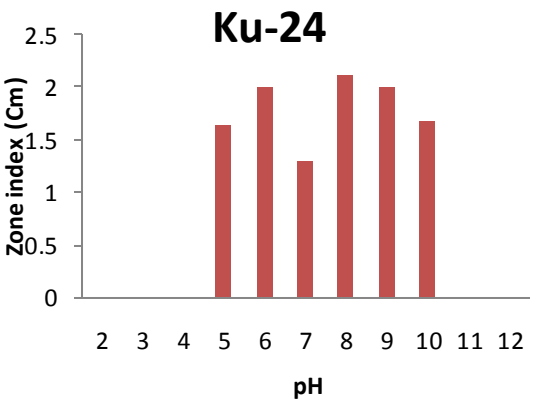

Ku-19
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

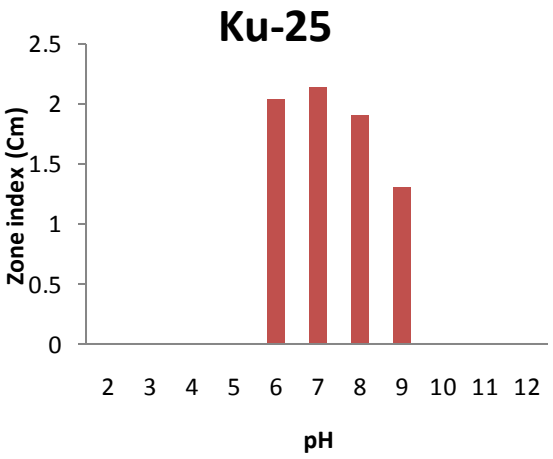

Ku-20
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

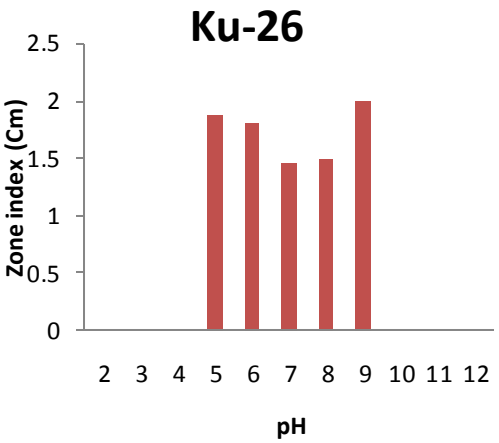

Ku-21
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

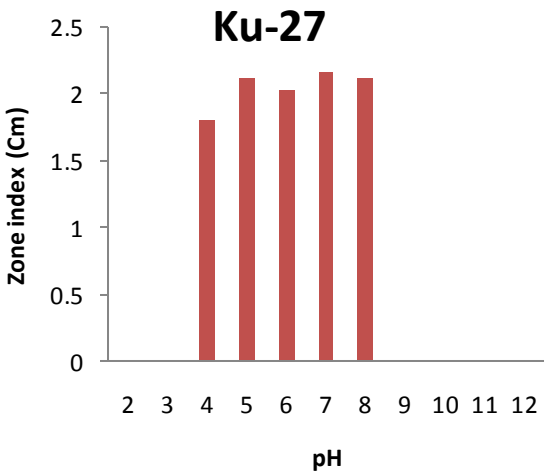

Ku-21
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

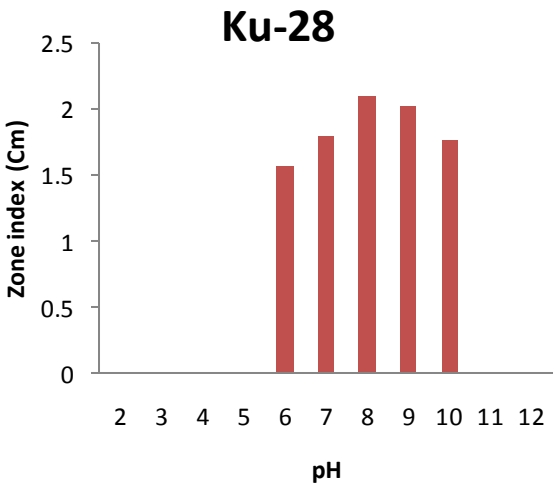

Ku-23
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

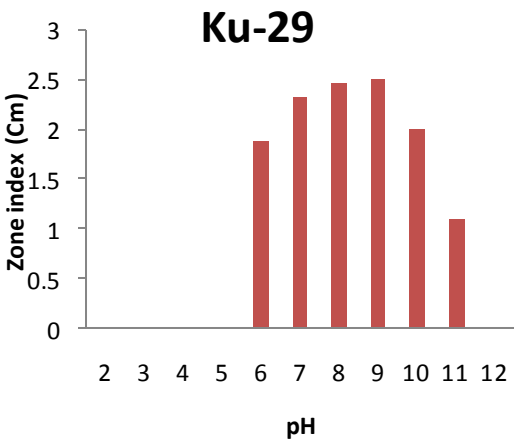

Ku-24
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

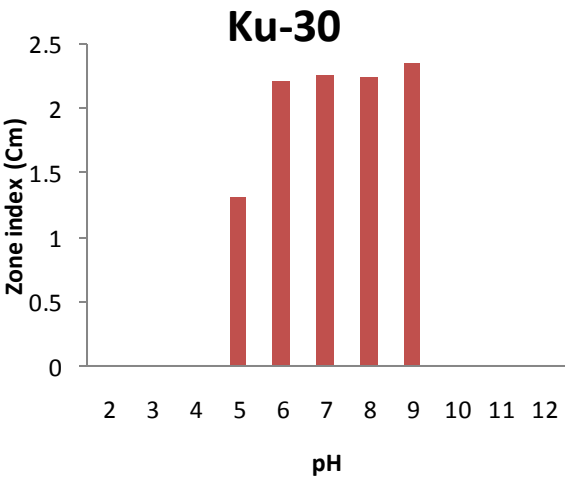

Ku-25
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH


Ku-26
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH


Ku-27
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

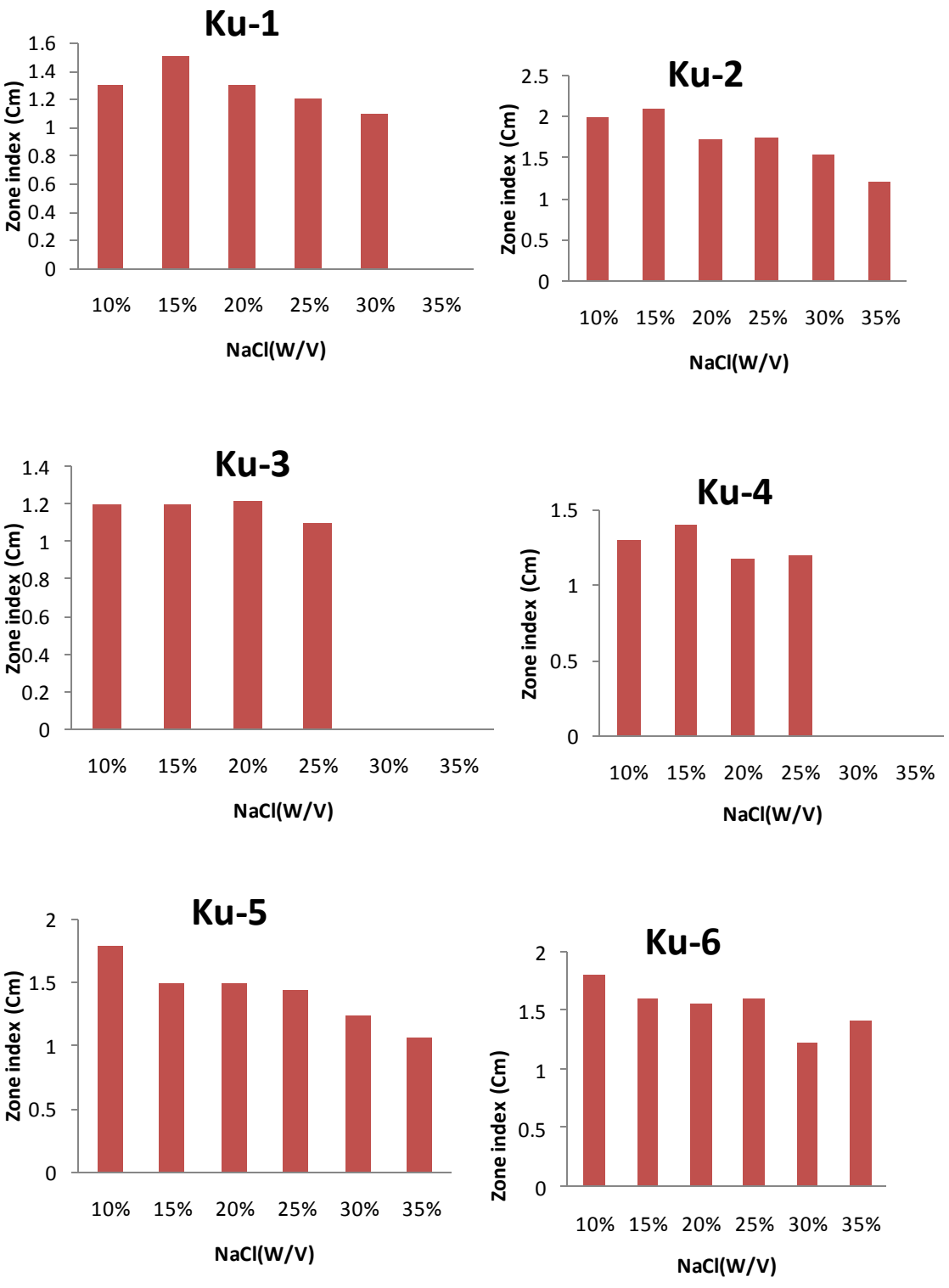

Ku-28
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

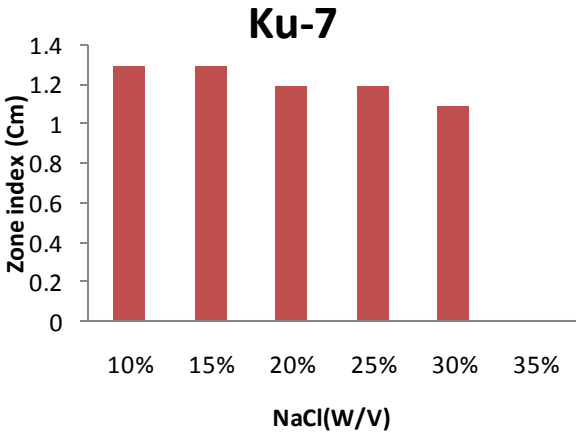

Ku-29
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

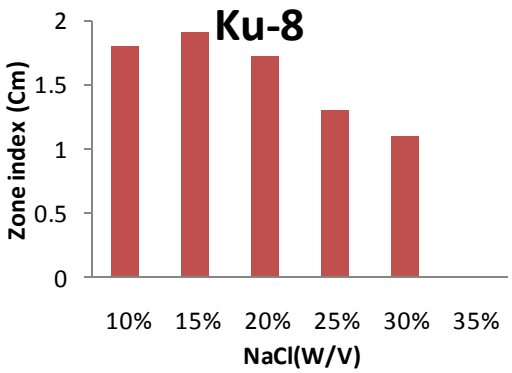

Ku-30
Zone index (Cm)
2.5
2
1.5
1
0.5
0
2 3 4 5 6 7 8 9 10 11 12
pH

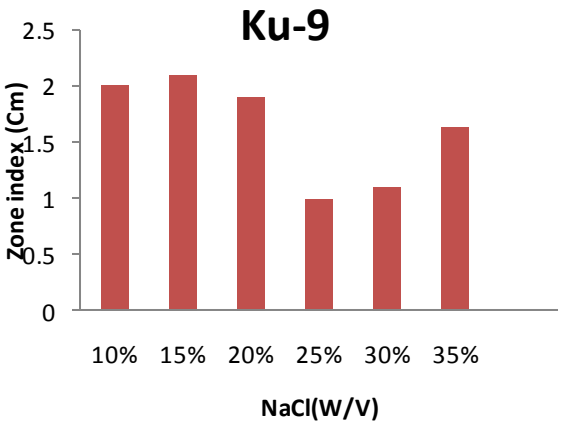
❖ All the isolates could grow at 10%- 35% NaCl concentration but majority of them produced highest lipase at 10% and 15% NaCl concentration (Graphs-4.22).

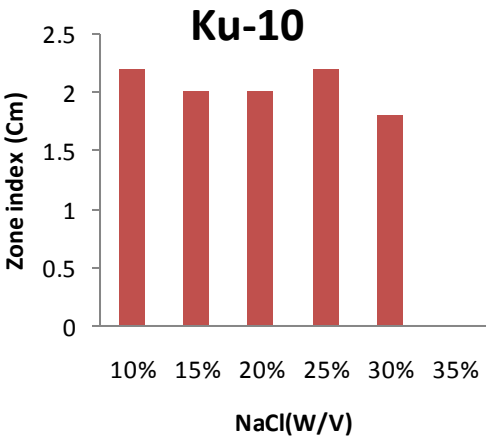
Graphs- 4.22 Effect of NaCl

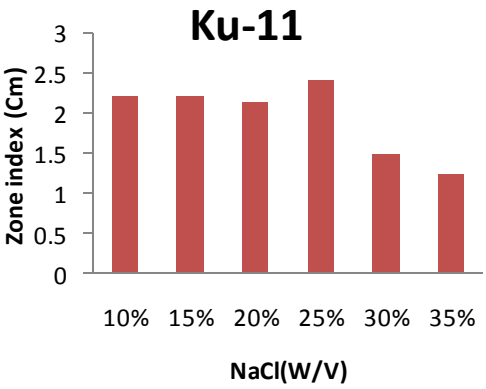


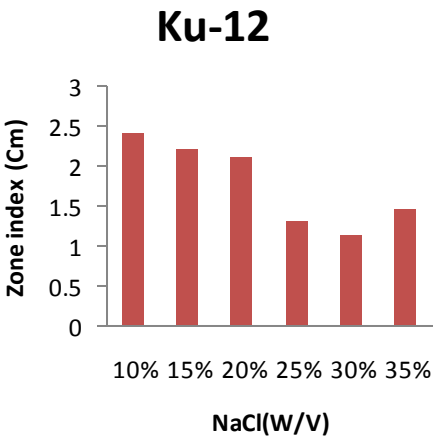

Ku-7
Zone index (Cm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

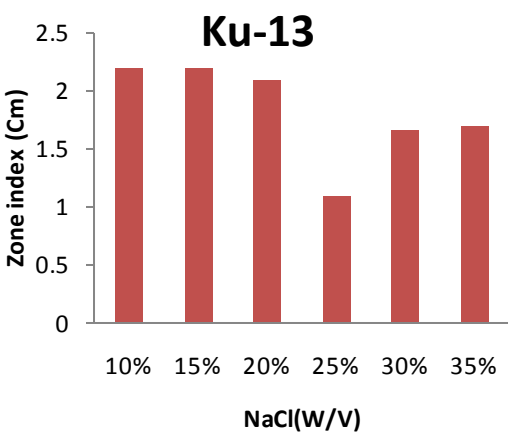

Ku-8
Zone index (Cm)
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

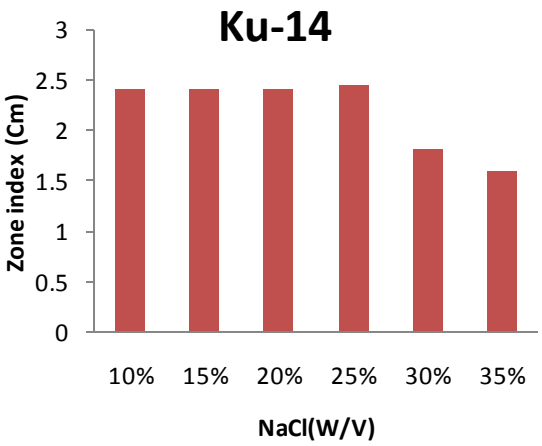

Ku-9
Zone index (Cm)
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

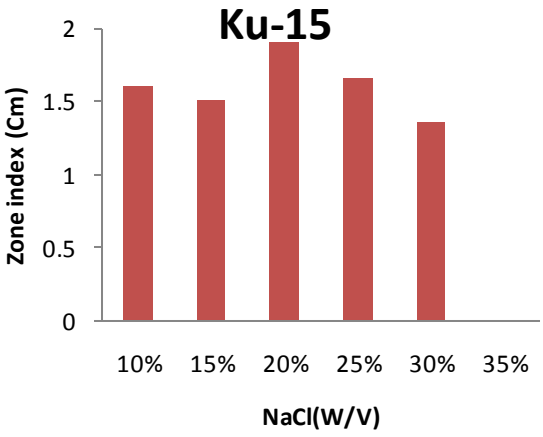

Ku-10
Zone index (Cm)
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

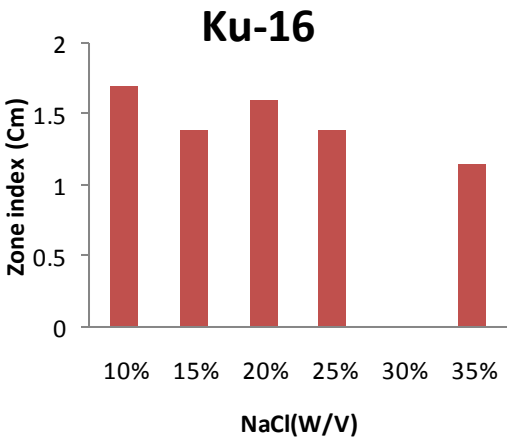

Ku-11
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

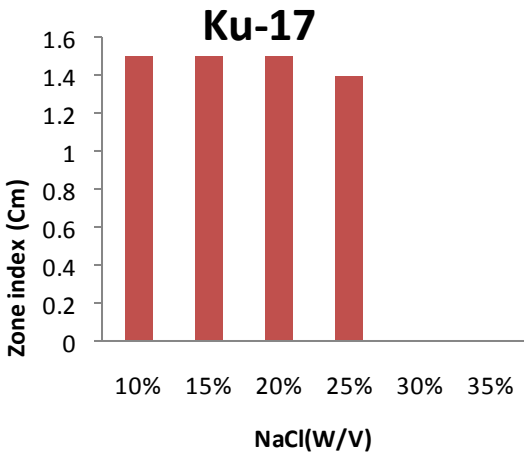

Ku-12
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

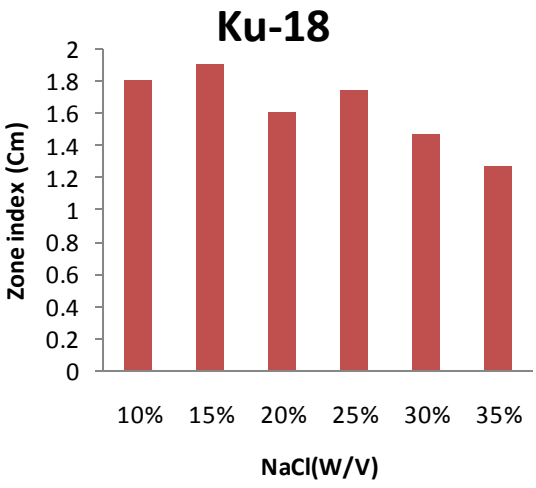

Ku-13
Zone index (Cm)
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

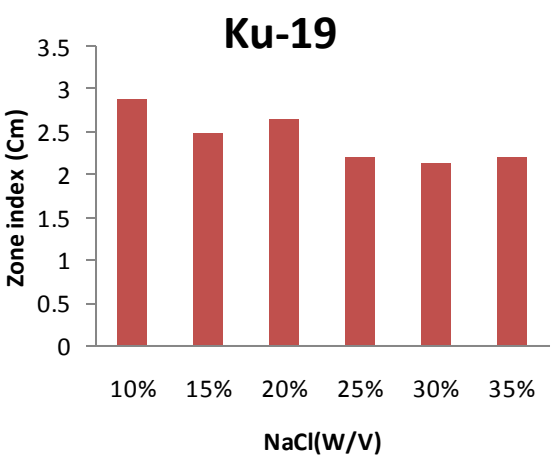

Ku-14
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

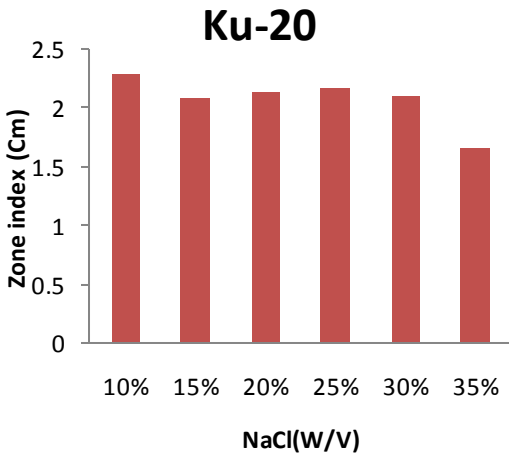

Ku-15
Zone index (Cm)
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

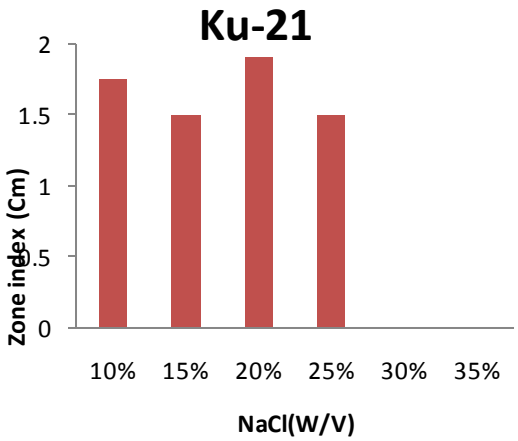

Ku-16
Zone index (Cm)
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

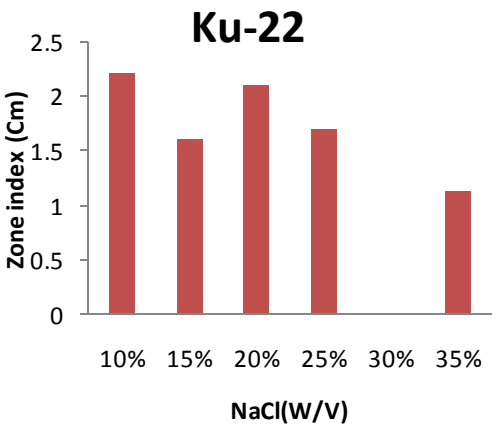

Ku-17
Zone index (Cm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

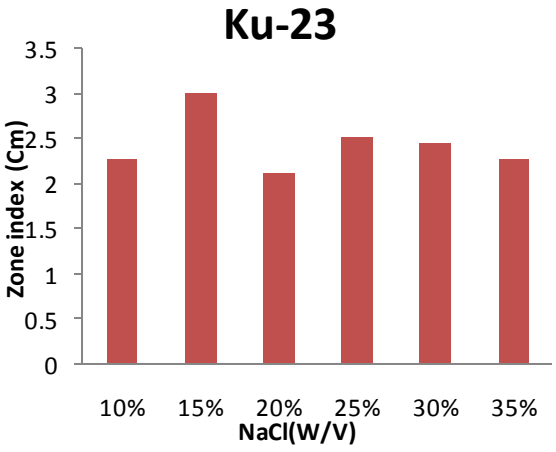

Ku-18
Zone index (Cm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

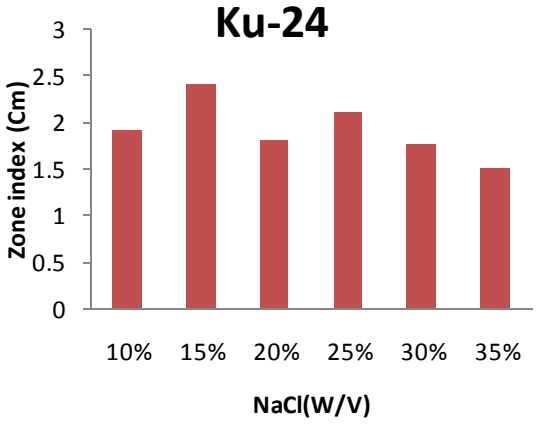

Ku-19
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

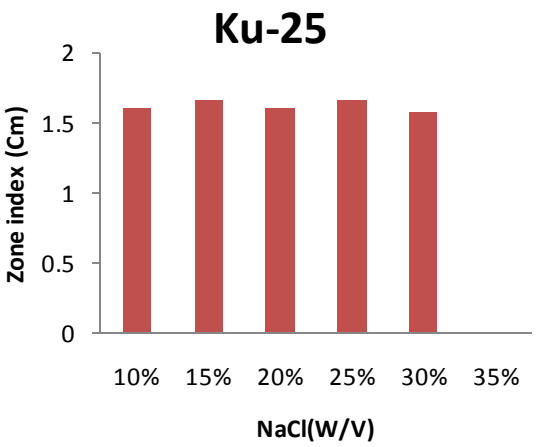

Ku-20
Zone index (Cm)
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

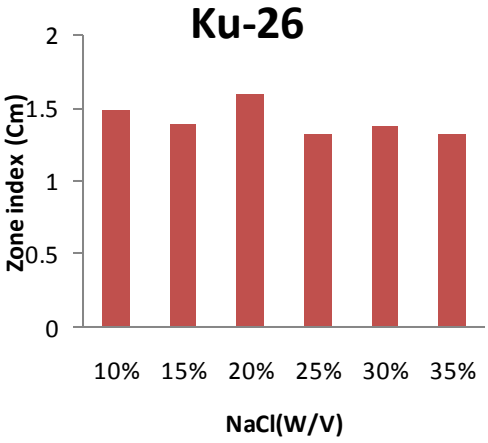

Ku-21
Zone index (Cm)
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

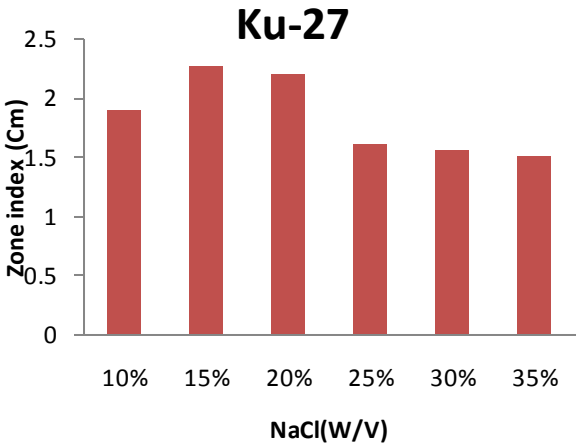

Ku-22
Zone index (Cm)
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

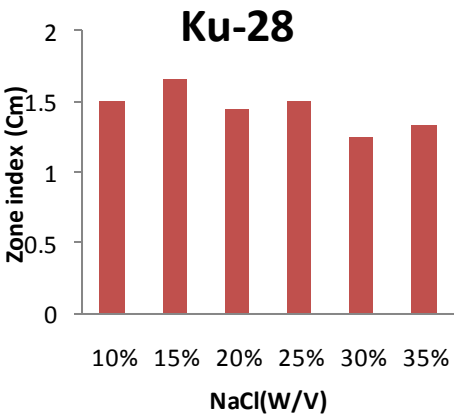

Ku-23
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

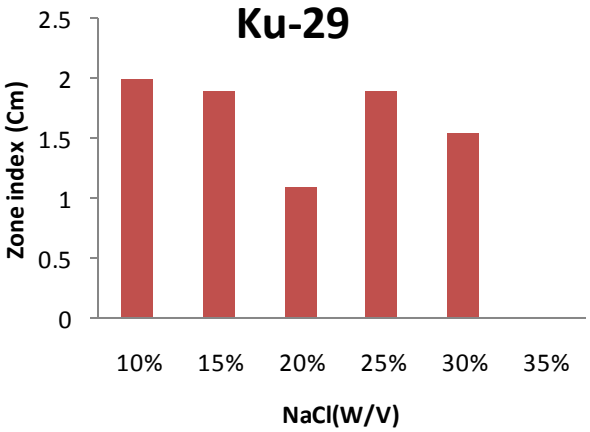

Ku-24
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
10%
15%
20%
25%
30%
35%
NaCl(W/V)

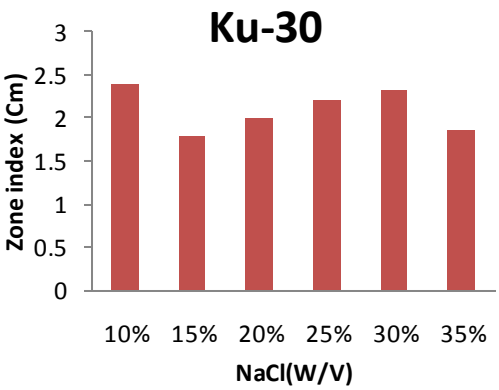






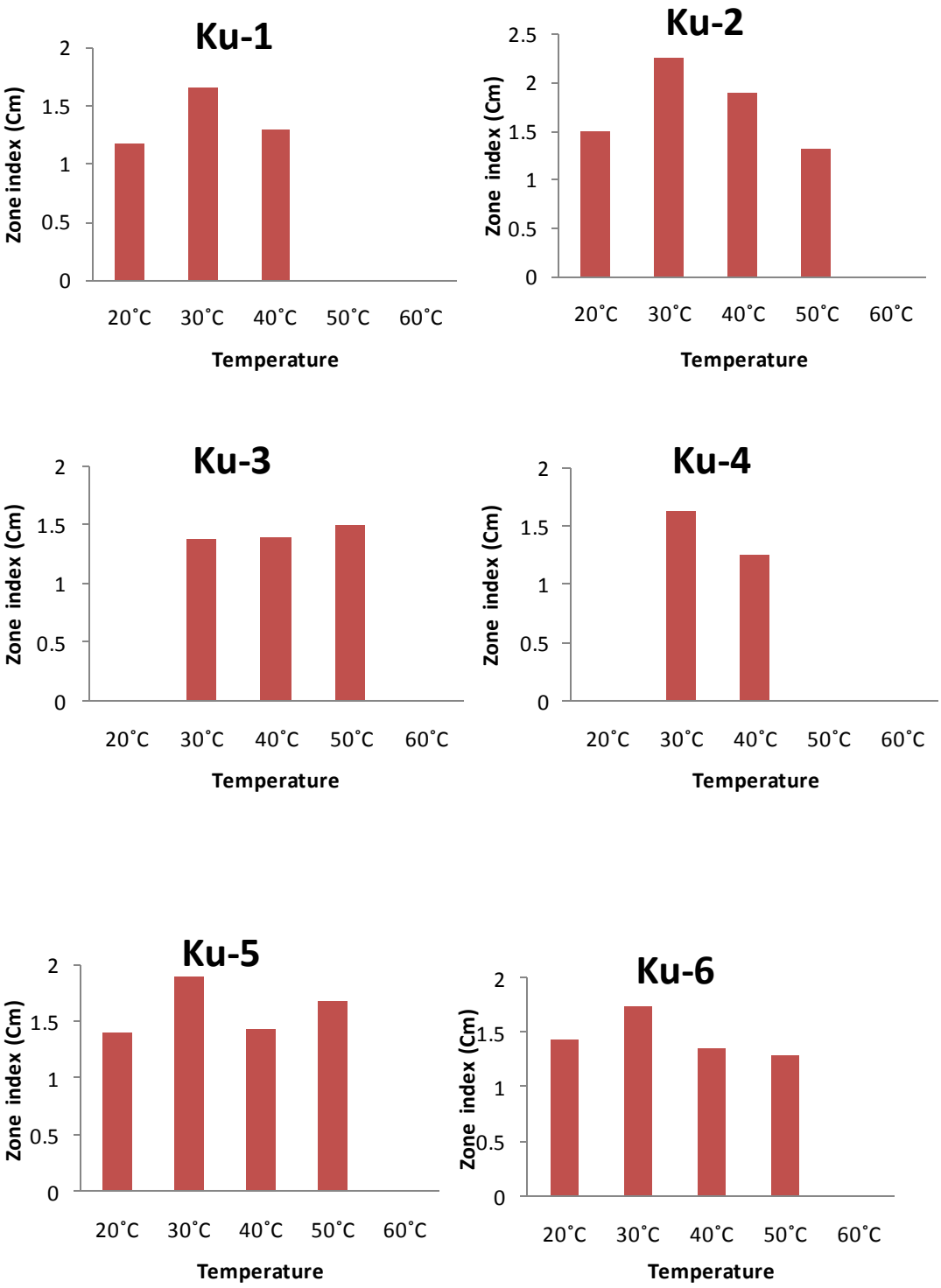


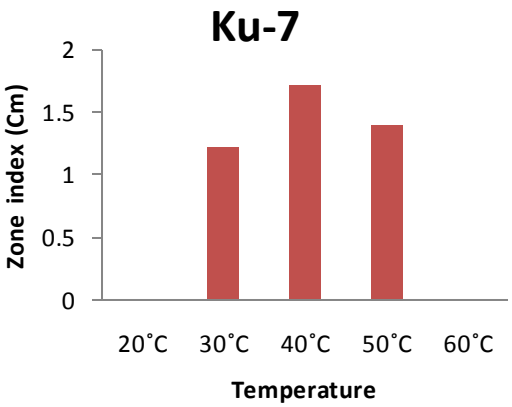


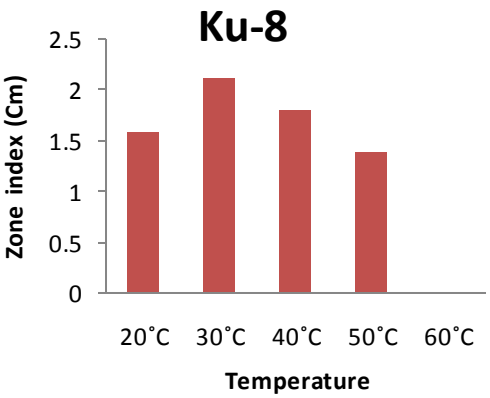


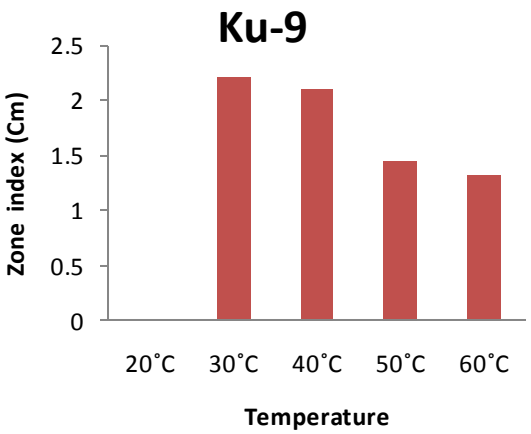
- Extreme halophiles preferred to grow and produce highest lipase at 30˚C and 40˚C temperatures. Very few isolates could grow at 60˚C (Graphs- 4.23).

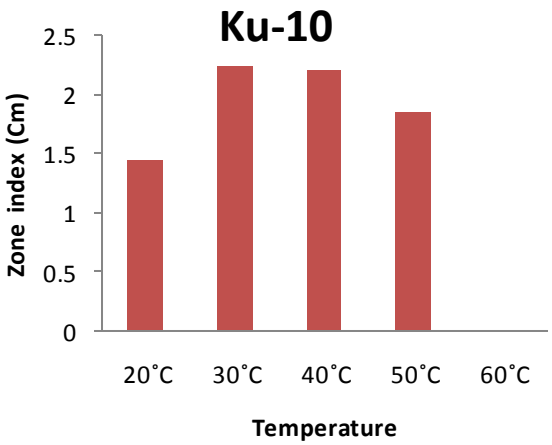
Graphs 4.23 Effect of Temperature

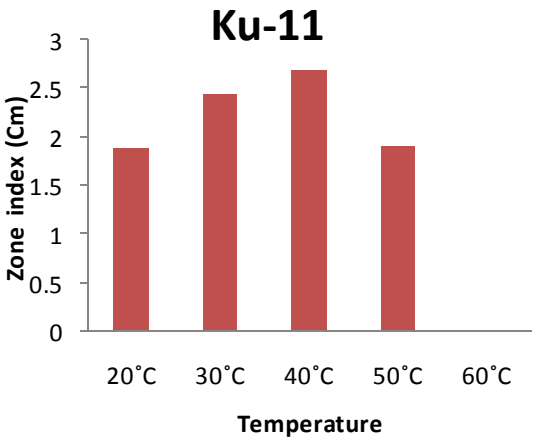


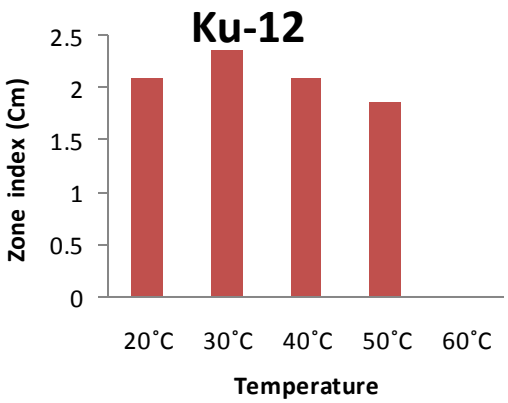

Ku-7
Zone index (Cm)
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

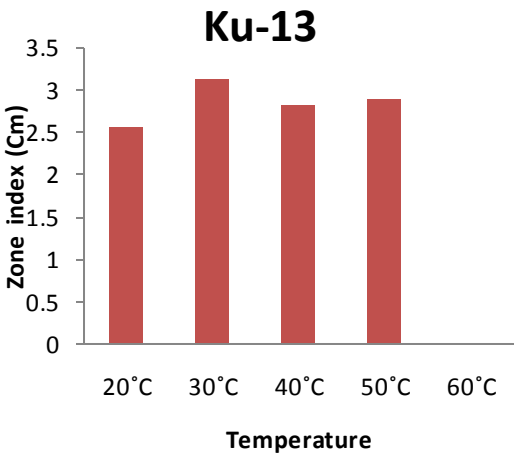

Ku-8
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

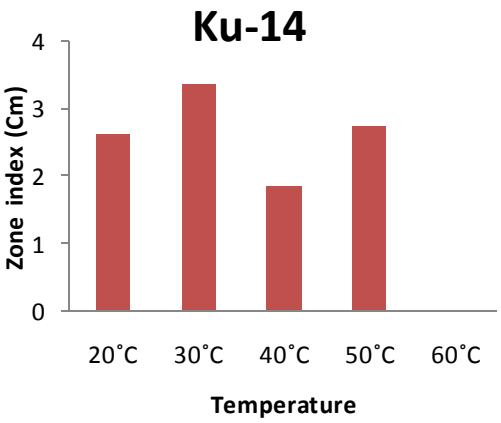

Ku-9
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

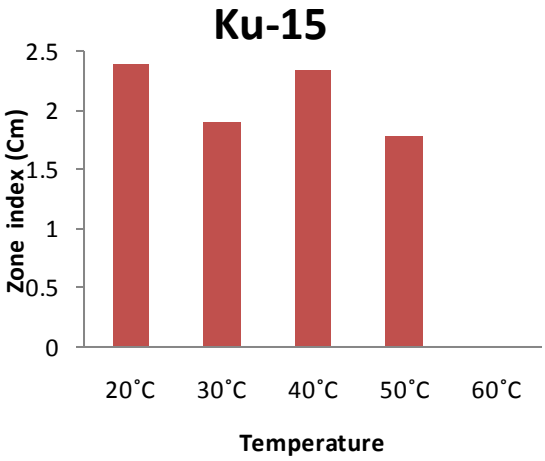

Ku-10
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

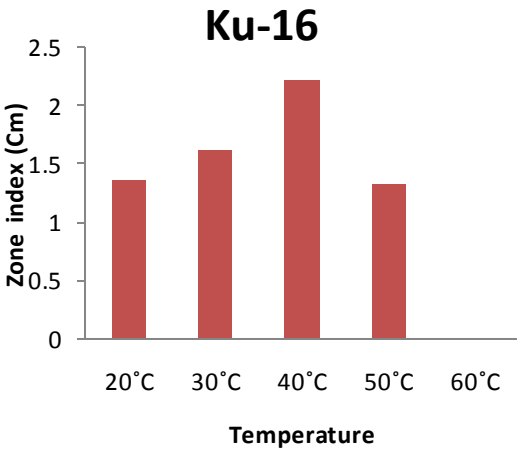

Ku-11
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

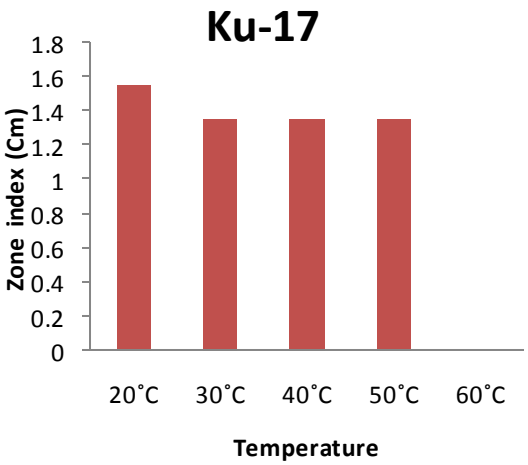

Ku-12
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

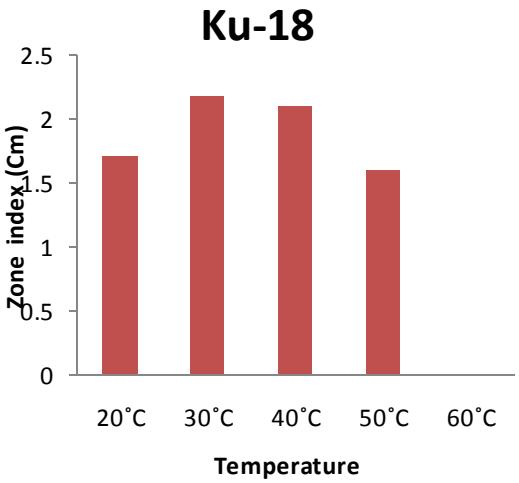

Ku-13
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

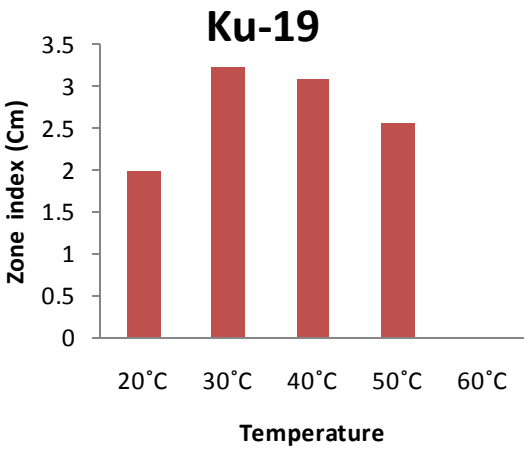

Ku-14
Zone index (Cm)
4
3
2
1
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

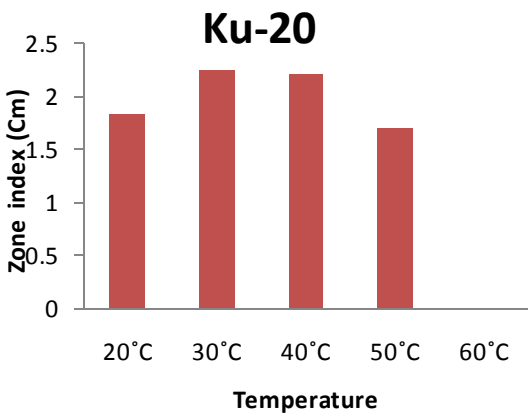

Ku-15
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

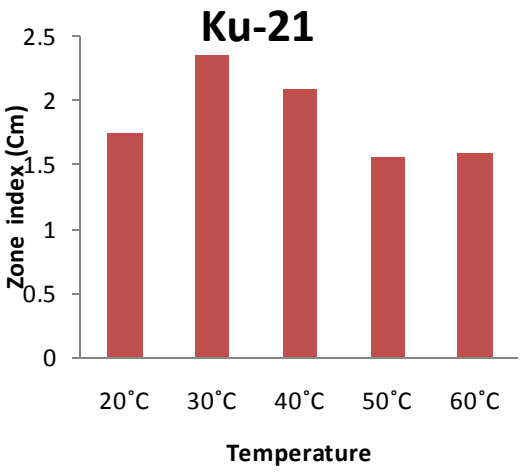

Ku-16
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

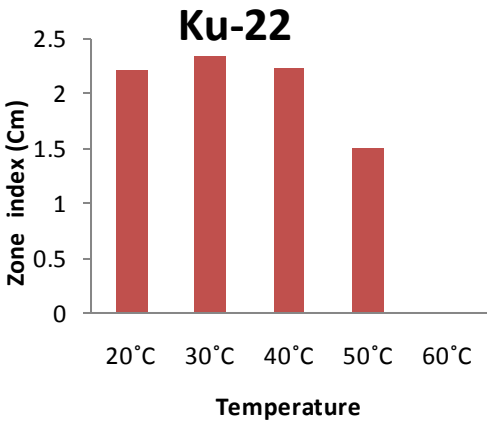

Ku-17
Zone index (Cm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

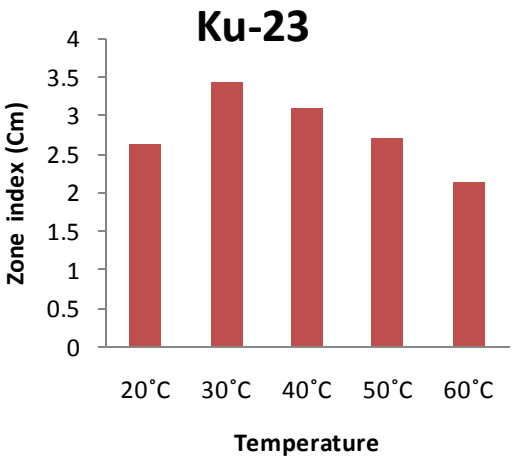

Ku-18
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

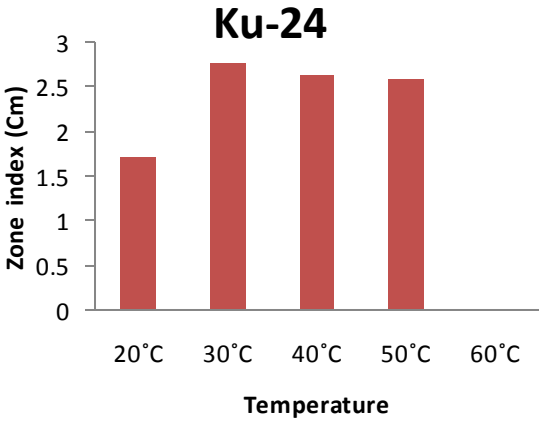

Ku-19
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

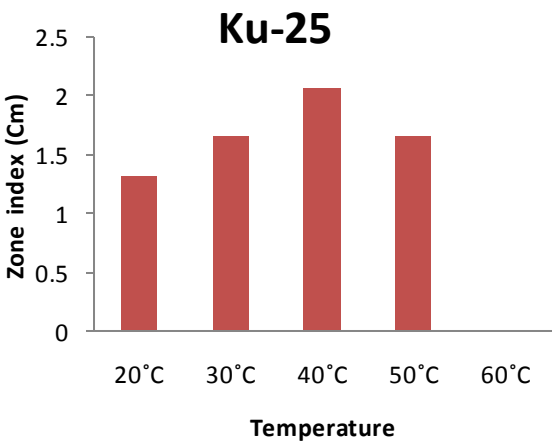

Ku-20
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

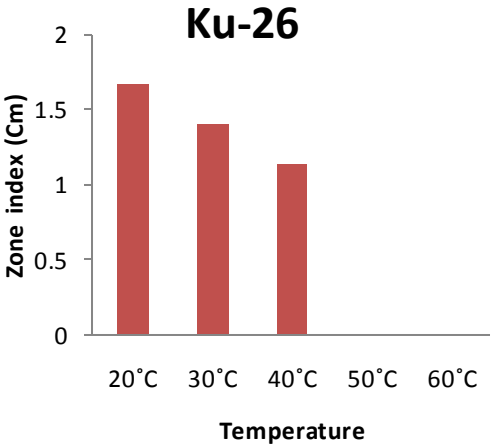

Ku-21
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

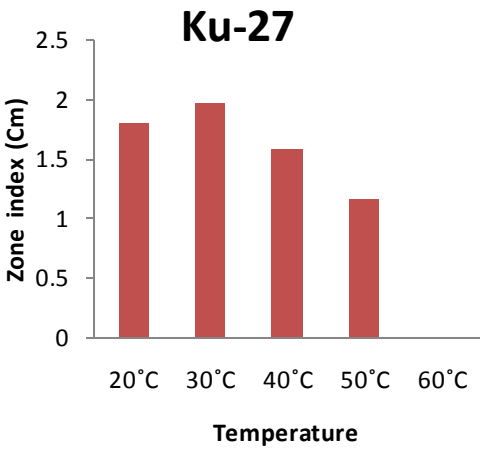

Ku-22
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

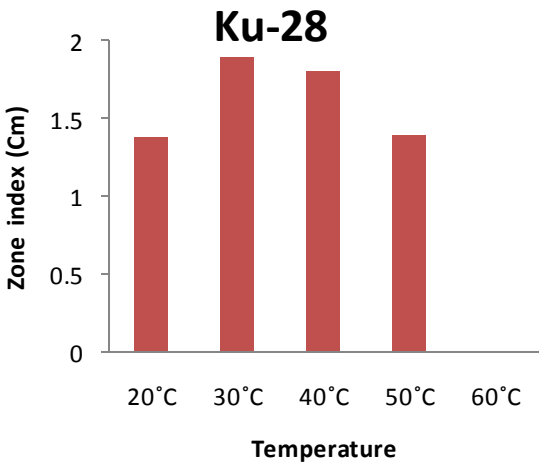

Ku-23
Zone index (Cm)
4
3.5
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

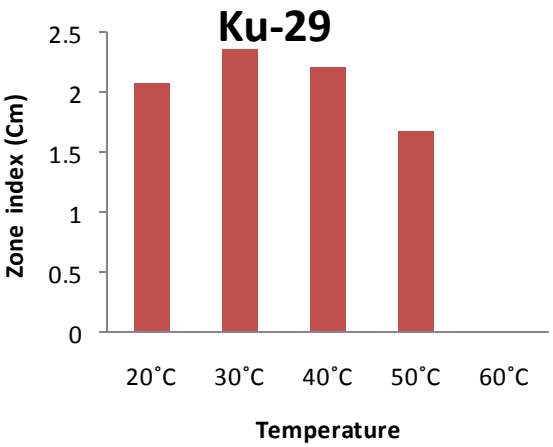

Ku-24
Zone index (Cm)
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Ku-25
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Ku-26
Zone index (Cm)
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Ku-27
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Ku-28
Zone index (Cm)
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature


Ku-29
Zone index (Cm)
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

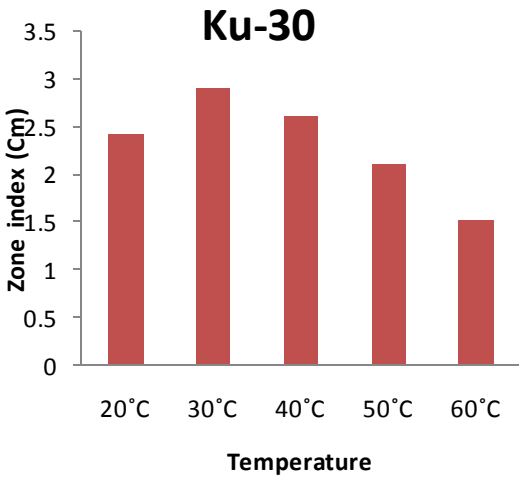

Ku-30
Zone index (Cm)
3.5
3
2.5
2
1.5
1
0.5
0
20˚C
30˚C
40˚C
50˚C
60˚C
Temperature

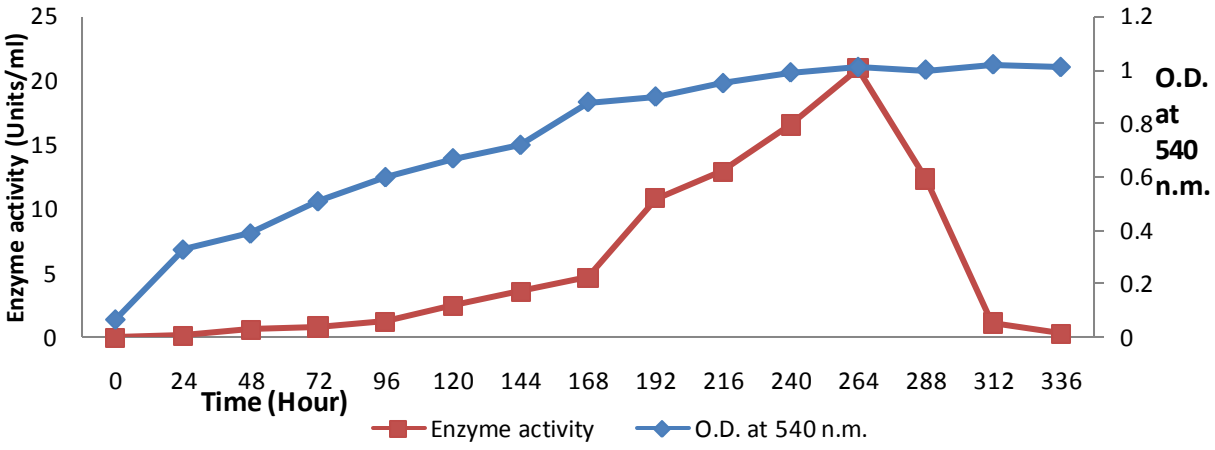
Table-4.22 Optimum and range of pH, Salt and temperature requirement for lipase production from extreme Halophiles

Sr. No	Isolates	pH		Salt (%)		Temperature (˚C)	
		Optimum	Range	Optimum	Range	Optimum	Range
1	Ku-1	10	5-10	15	10-30	30	20-40
2	Ku-2	7	4-10	15	10-35	30	20-50
3	Ku-3	4	4-10	20	10-25	50	30-50
4	Ku-4	8	4-10	15	10-25	30	30-40
5	Ku-5	6	4-9	10	10-35	30	20-50
6	Ku-6	7	5-9	10	10-35	30	20-50
7	Ku-7	7	4-9	15	10-30	40	30-50
8	Ku-8	7	5-9	15	10-30	30	20-50
9	Ku-9	7	5-11	15	10-35	30	30-60
10	Ku-10	7	5-10	10, 25	10-30	30-40	20-50
11	Ku-11	7	4-9	25	10-35	40	20-50
12	Ku-12	8	5-9	10	10-35	30	20-50
13	Ku-13	7	5-9	15	10-35	30	20-50
14	Ku-14	5	4-9	25	10-35	30	20-50
15	Ku-15	10	5-10	20	10-30	30	20-50
16	Ku-16	8	4-8	10	10-35	40	20-50
17	Ku-17	10	6-10	10-20	10-25	20	20-50
18	Ku-18	8	4-8	15	10-35	30	20-50
19	Ku-19	7	6-10	10	10-35	30	20-50
20	Ku-20	7	5-9	10	10-35	30	20-50
21	Ku-21	7	5-9	20	10-25	30	20-60
22	Ku-22	8	6-10	10	10-35	30	20-50
23	Ku-23	7	5-9	15	10-35	30	20-60
24	Ku-24	8	5-10	15	10-35	30	20-50
25	Ku-25	7	6-9	15, 25	10-30	40	20-50
26	Ku-26	9	5-9	20	10-35	20	20-40
27	Ku-27	7	4-8	15	10-35	30	20-50
28	Ku-28	8	6-10	15	10-35	30	20-50
29	Ku-29	9	6-11	10	10-30	30	20-50
30	Ku-30	9	5-9	10	10-35	30	20-60

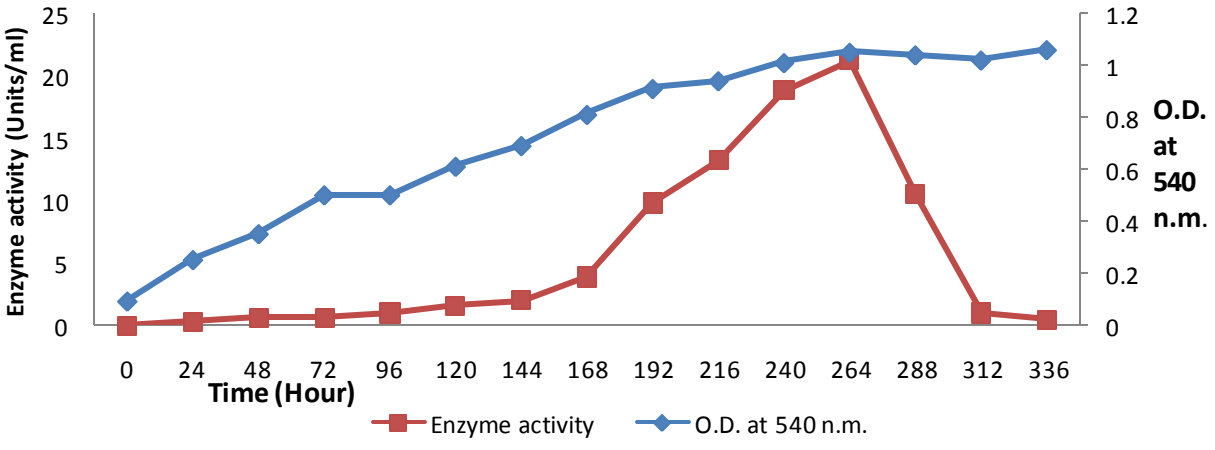
Effect of pH, salt, temperature and substrate concentration on lipase secretion in liquid media

- ❖ Three moderate and three extreme halophiles were selected on the basis of zone index for further study on lipase production in liquid media. Growth and lipase production pattern for all the isolates is shown in graph- 4.24-4.30

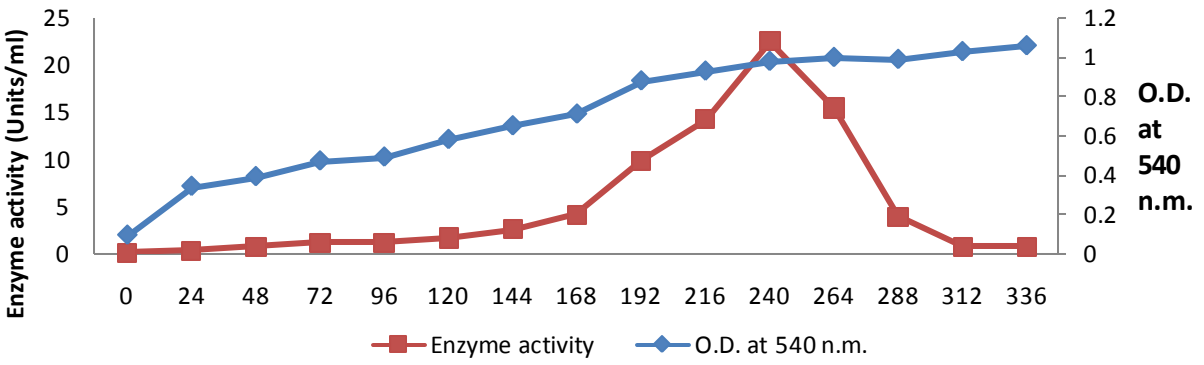
Graph 4.24 Isolate Mk-4



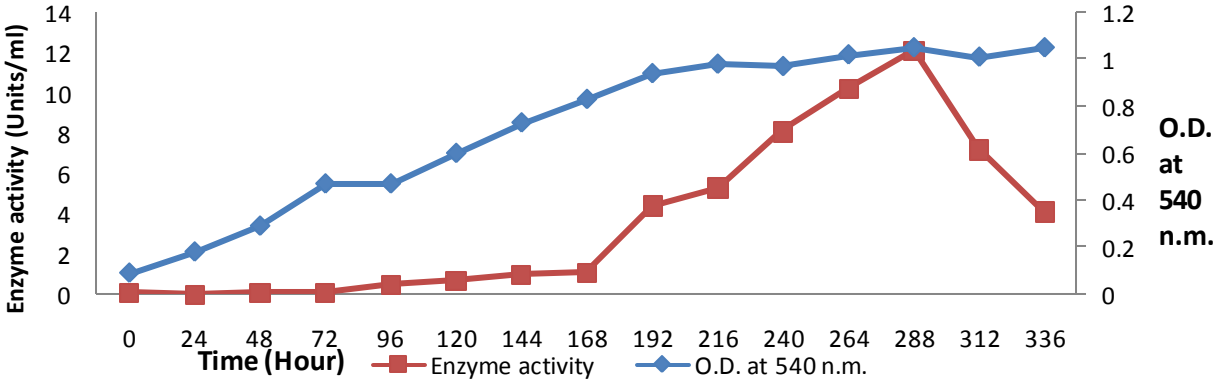
Graph 4.25 Isolate Mk-18



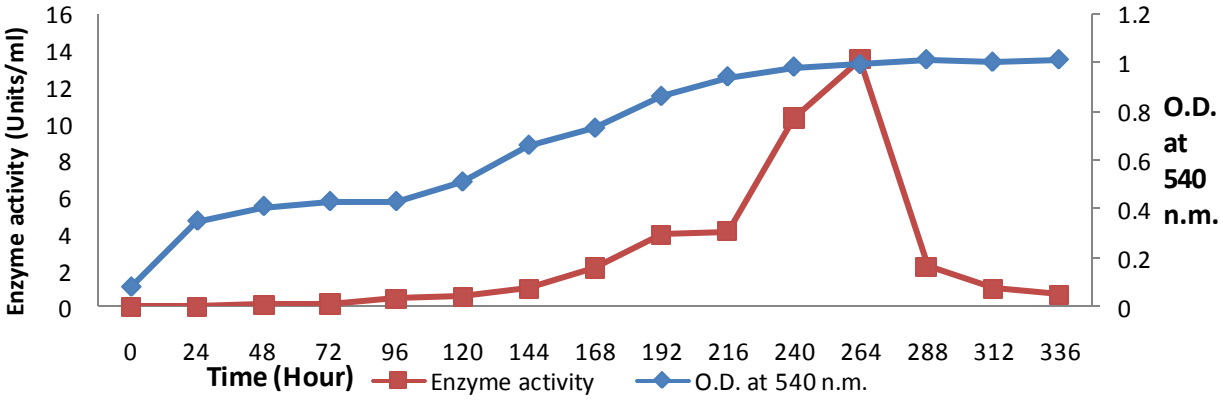
Graph 4.26 Isolate Mk-23



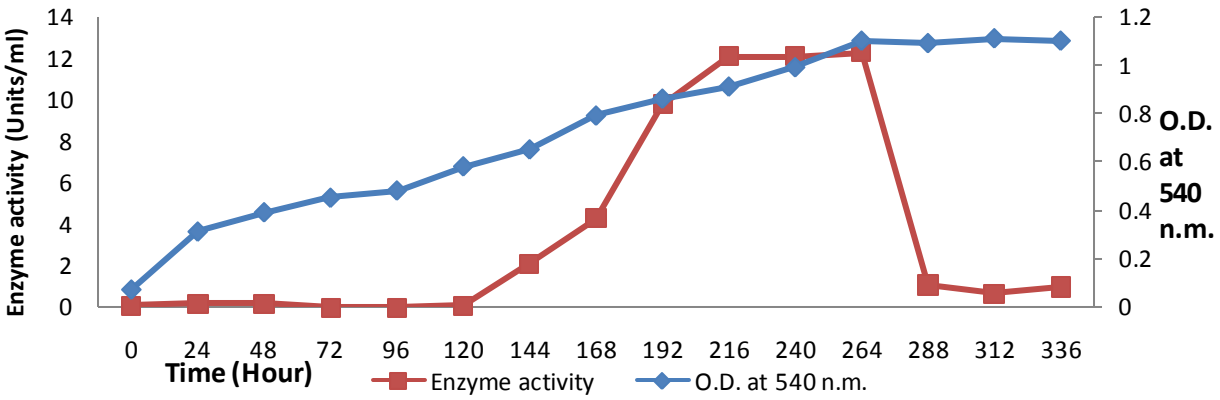
Graph 4.27 Isolate Ku-10



Graph 4.28 Isolate Ku-19

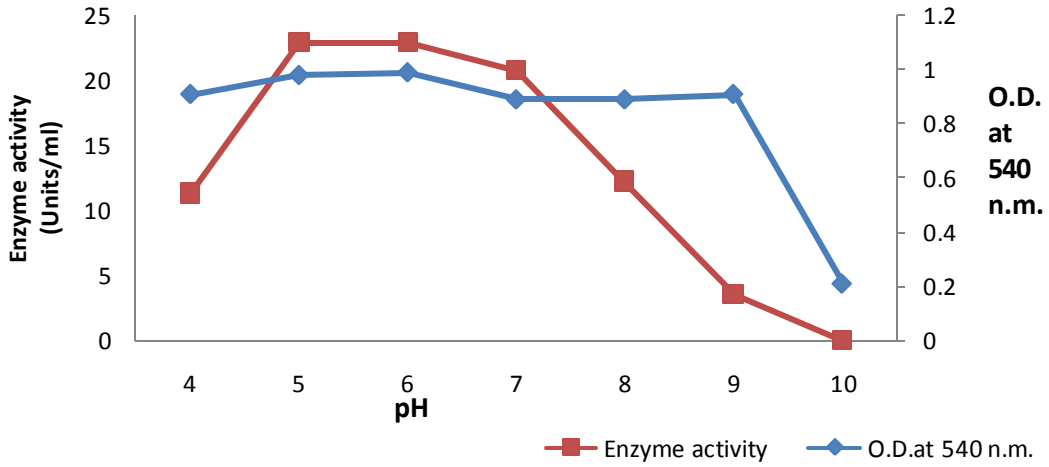


Graph 4.29 Isolate Ku-20

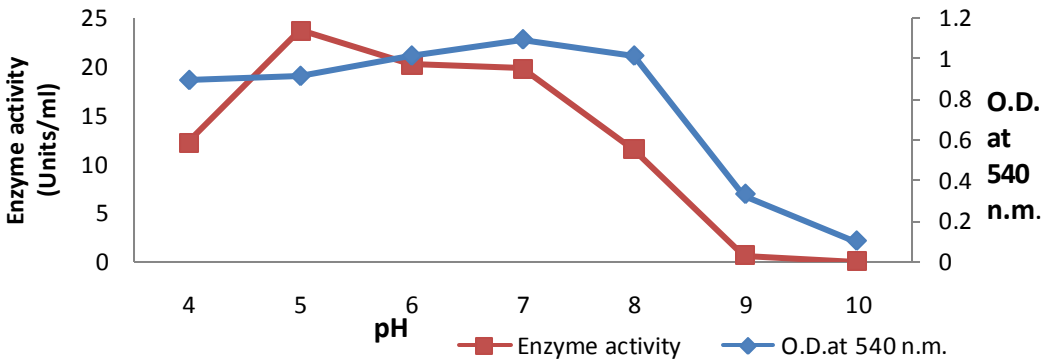


- Modrate halophilic isolate preferred to grow and produced lipase in the pH range of 5-8 while extreme halophilic isolates preferred pH range of 5-10 for growth and 7-10 for lipase production. (Graph-4.30 to 4.35).

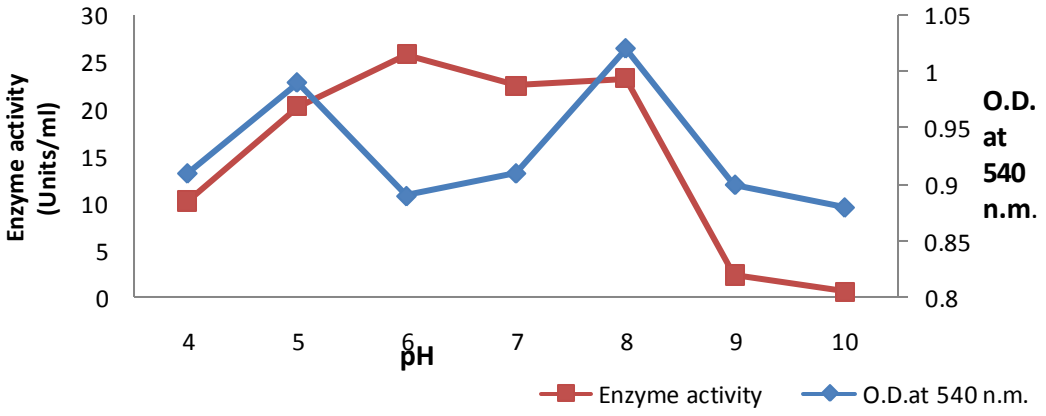
Graph 4.30 Effect of pH on Mk-4



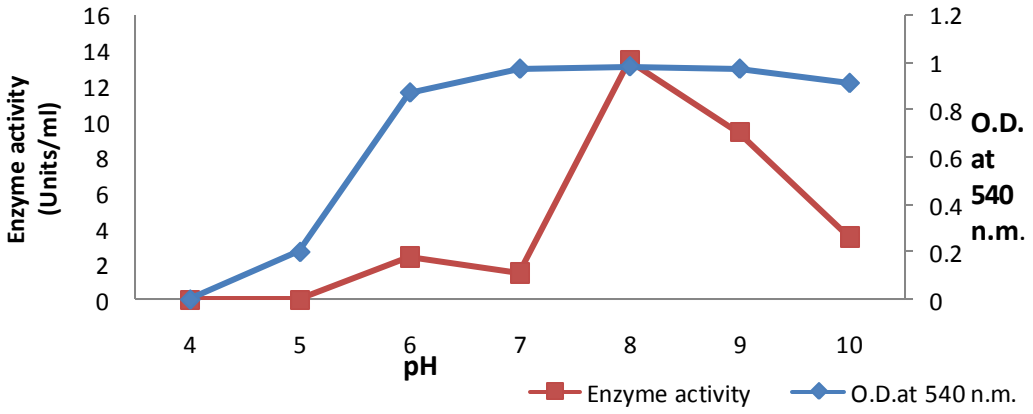
Graph 4.31 Effect of pH on Mk-18



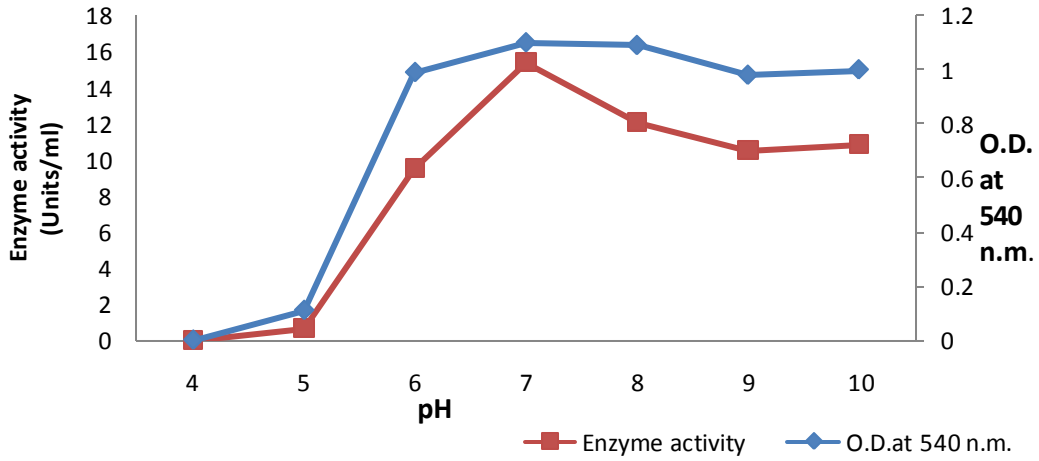
Graph 4.32 Effect of pH on Mk-23



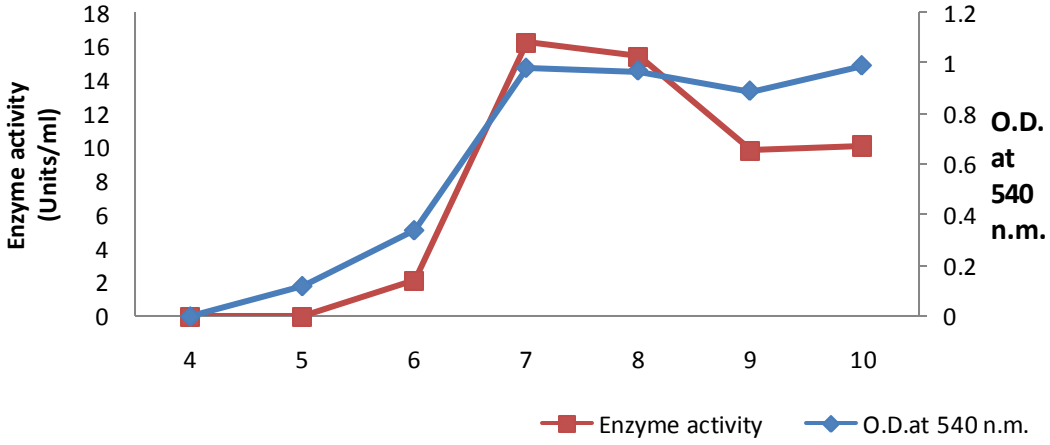
Graph 4.33 Effect of pH on Ku-10



Graph 4.34 Effect of pH on Ku-19

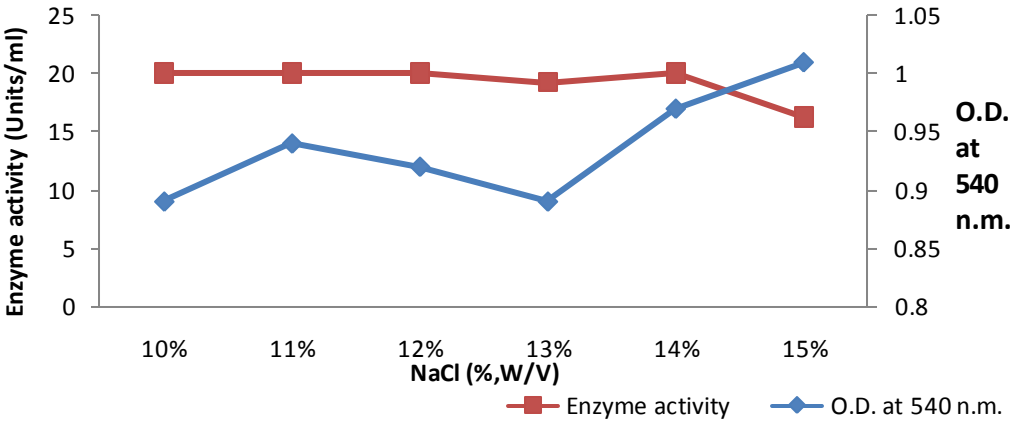


Graph 4.35 Effect of pH on Ku-20

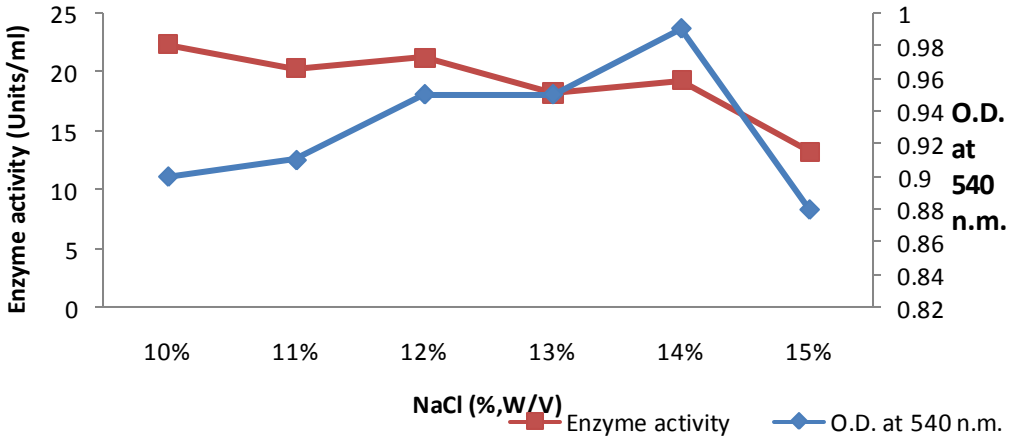


- Modrate halophilic isolate preferred to grow and produced lipase at salt concentration of 10-15**%** with 10-11% being optimum. while extreme halophilic isolates preferred salt concentration of 10-35% for growth with 10-15% being optimum for lipase production (Graph-4.36 to 4.41).

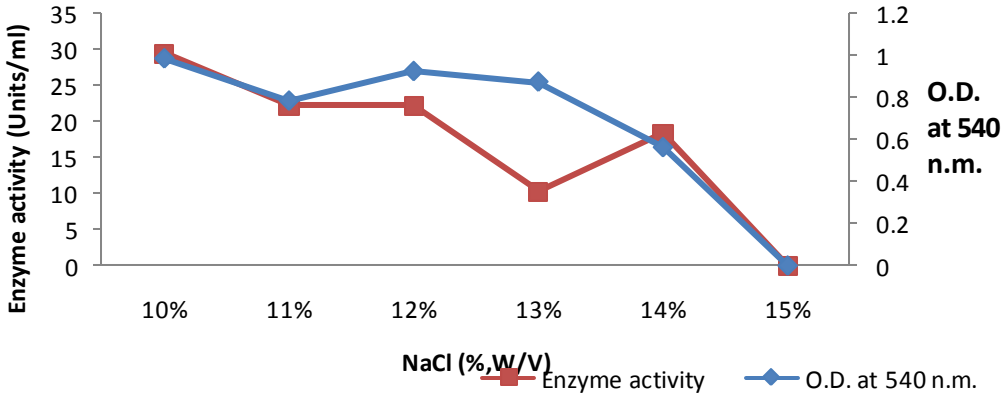
Graph 4.36 Effect of NaCl on Mk-4



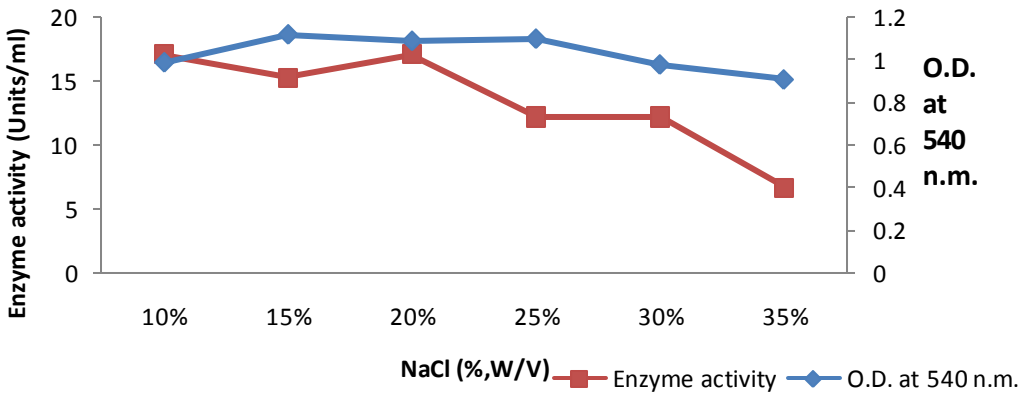
Graph 4.37 Effect of NaCl on Mk-18



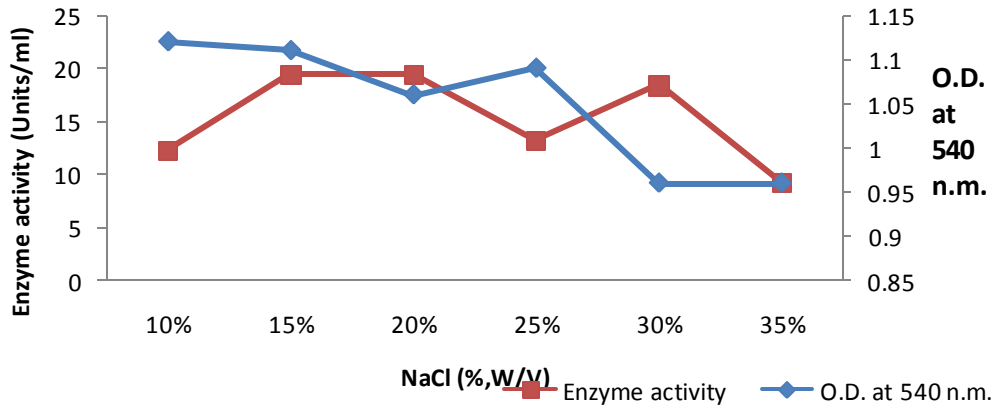
Graph 4.38 Effect of NaCl on Mk-23



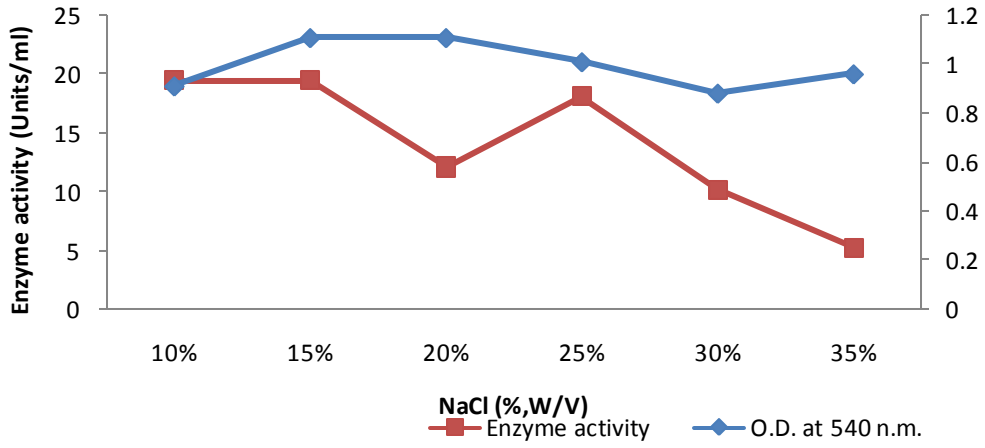
Graph 4.39 Effect of NaCl on Ku-10



Graph 4.40 Effect of NaCl on Ku-19

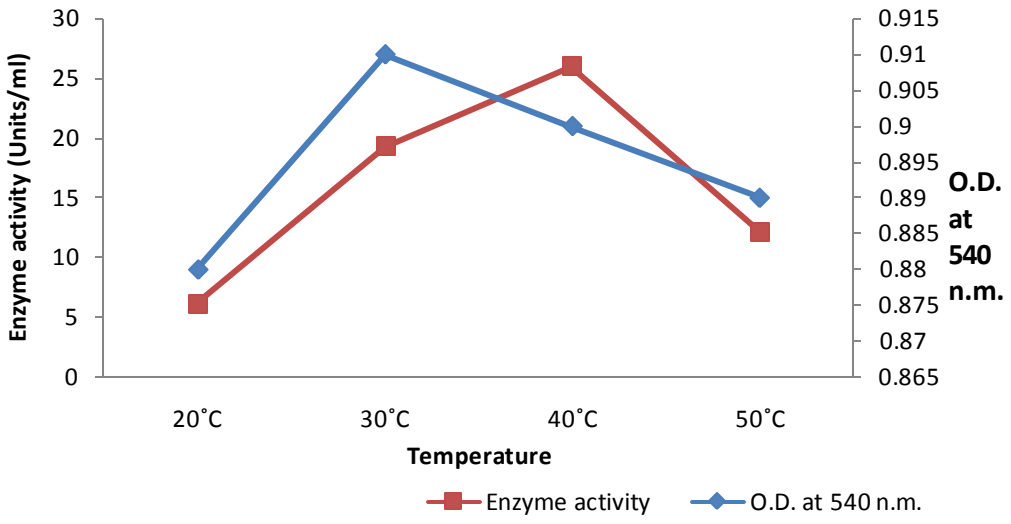


Graph 4.41 Effect of NaCl on Ku-20

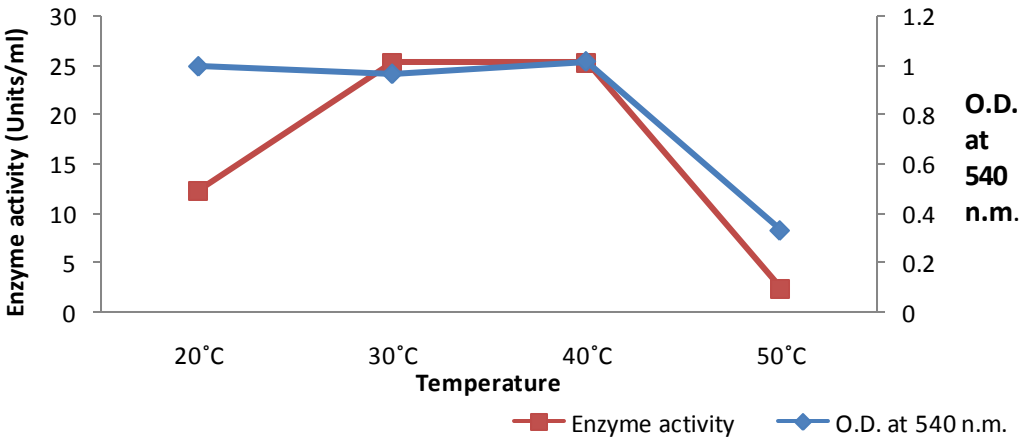


❖ Modrate halophilic isolate preferred to grow and produced lipase at at 30˚C. while extreme halophilic isolates preferred 30-40˚C for growth and lipase production (Graph-4.42 to 4.47).

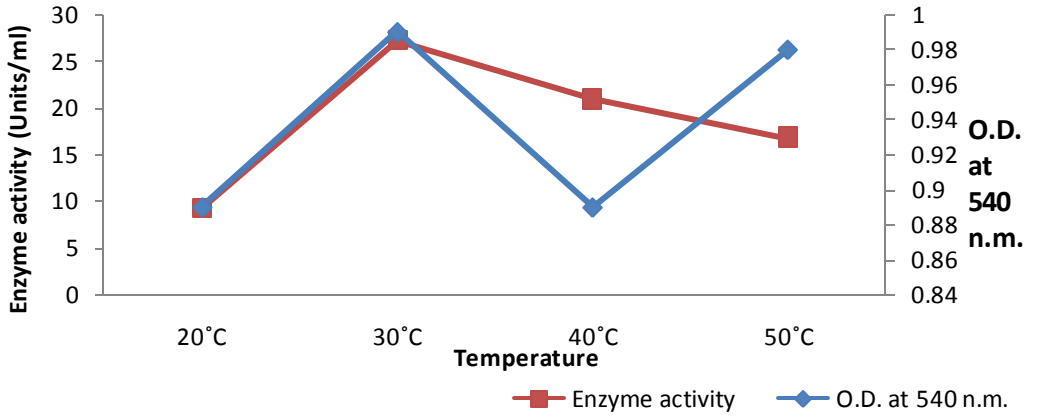
Graph 4.42 Effect of temperature on Mk-4



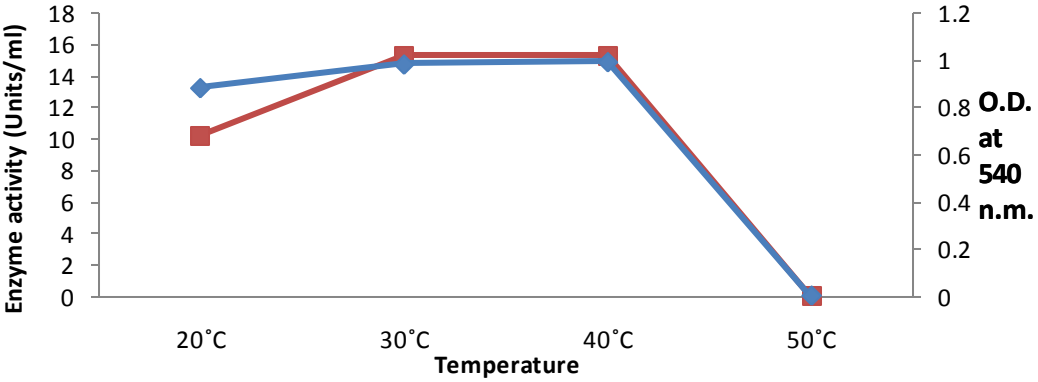
Graph 4.43 Effect of temperature on Mk-18



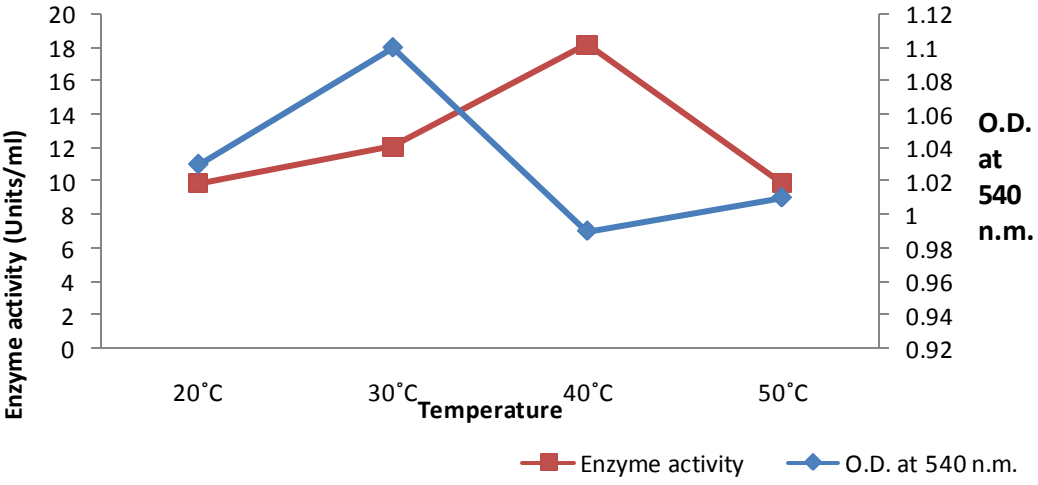
Graph 4.44 Effect of temperature with on Mk-23



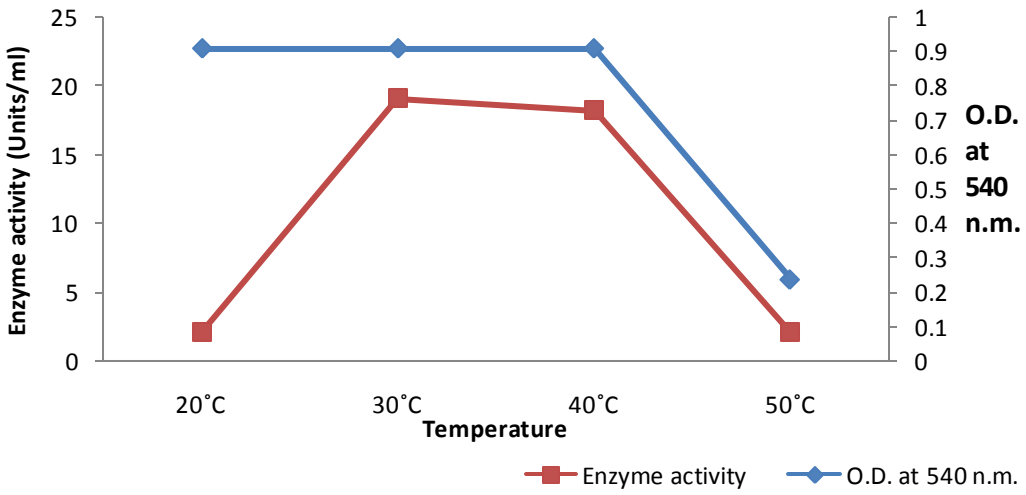
Graph 4.45 Effect of temperature on Ku-10



Graph 4.46 Effect of temperature with on Ku-19

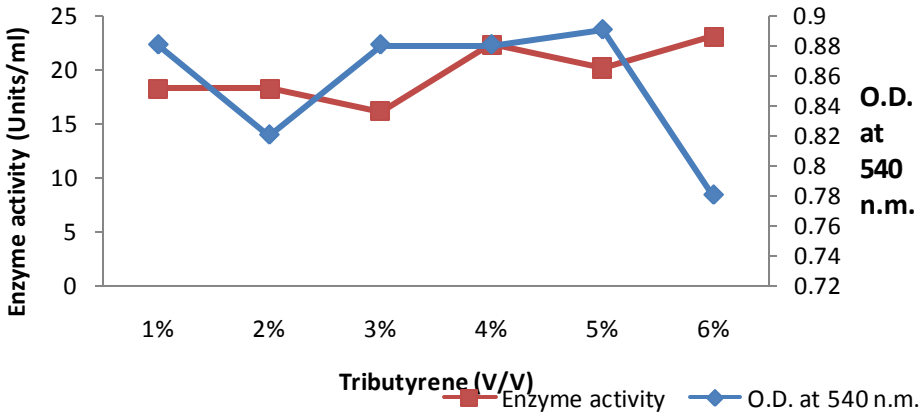


Graph 4.47 Effect of temperature on Ku-20

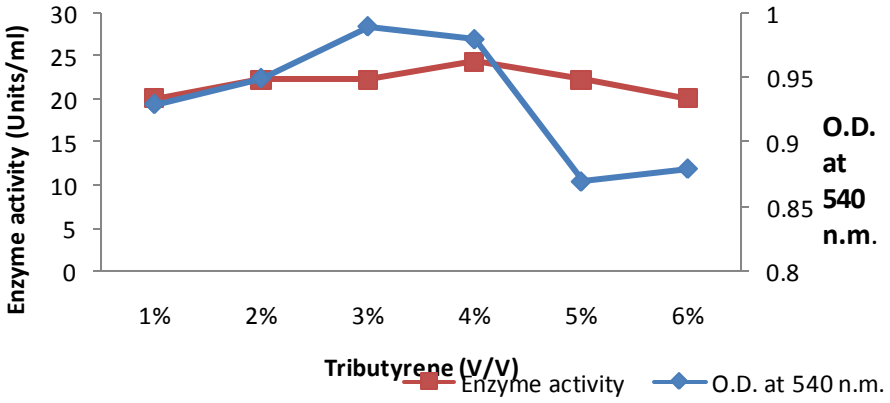


❖ Modrate halophilic isolate preferred to grow at 3-5% tributyrene concentration while optimum lipase production was found at 4-6%. Extreme halophilic isolates preferred to grow at 1-6% tributyrene while 4-6% tributyrene for lipase production (Graph-4.48 to 4.53).

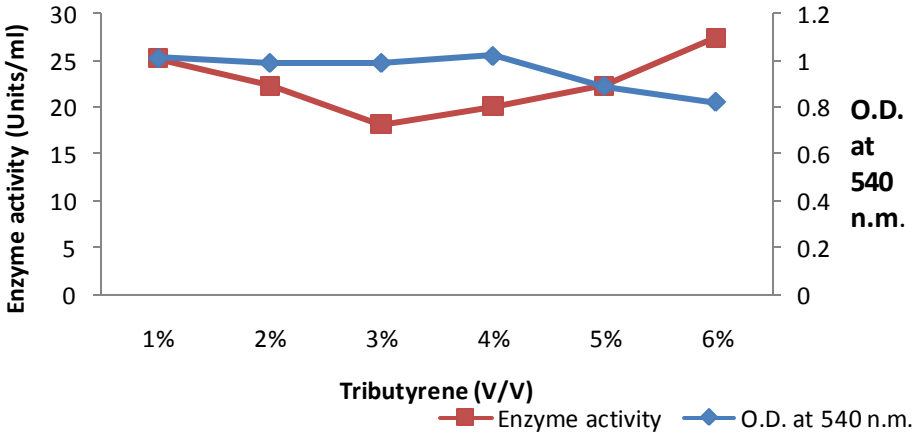
Graph 4.48 Effect of tributyrene on Mk-4



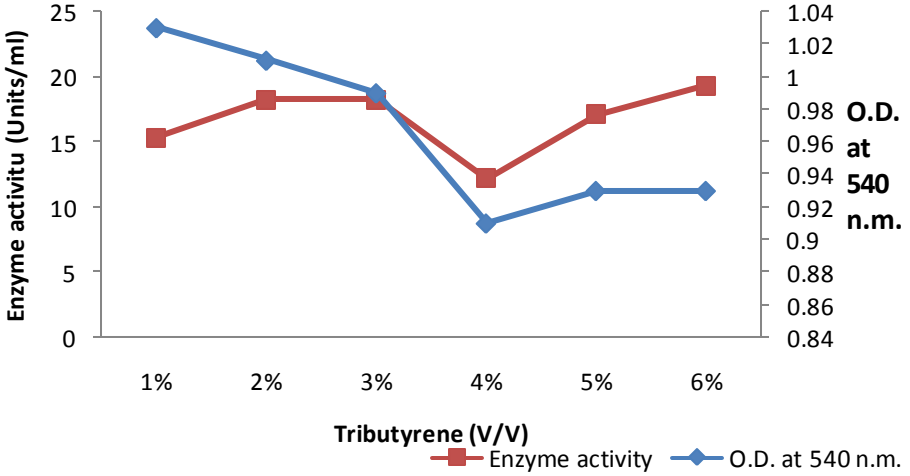
Graph 4.49 Effect of tributyrene on Mk-18



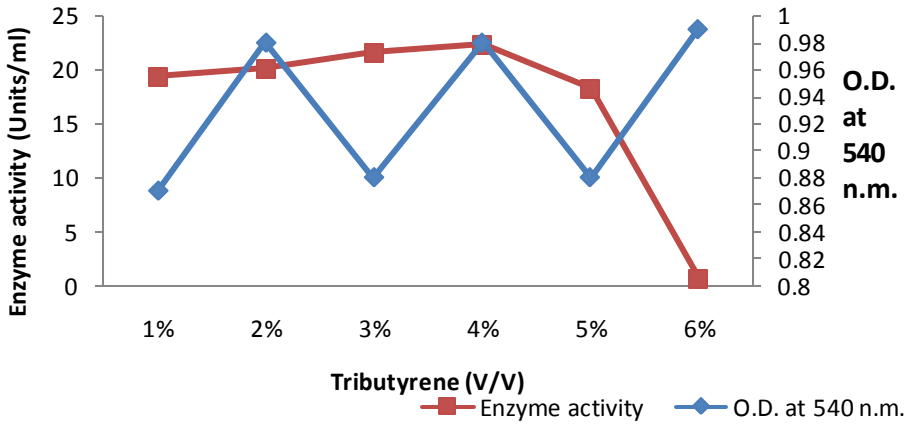
Graph 4.50 Effect of tributyrene on Mk-23



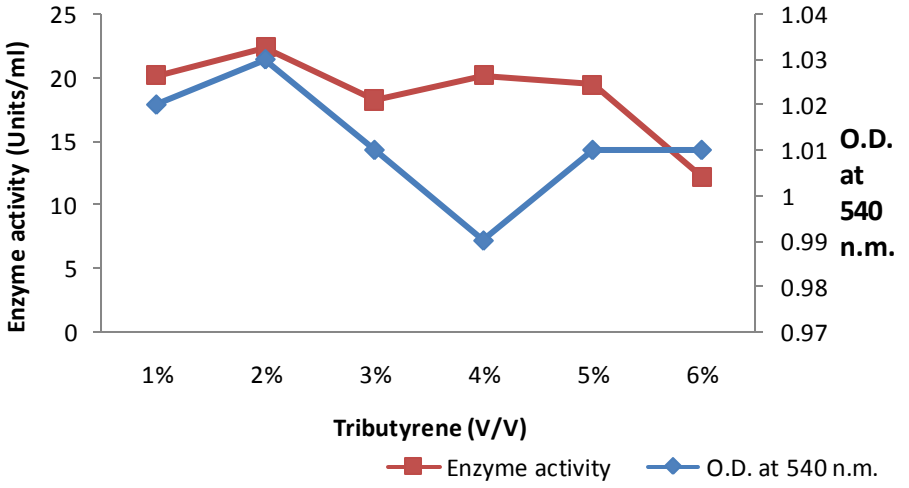
Graph 4.51 Effect of tributyrene on Ku-10



Graph 4.52 Effect of tributyrene on Ku-19



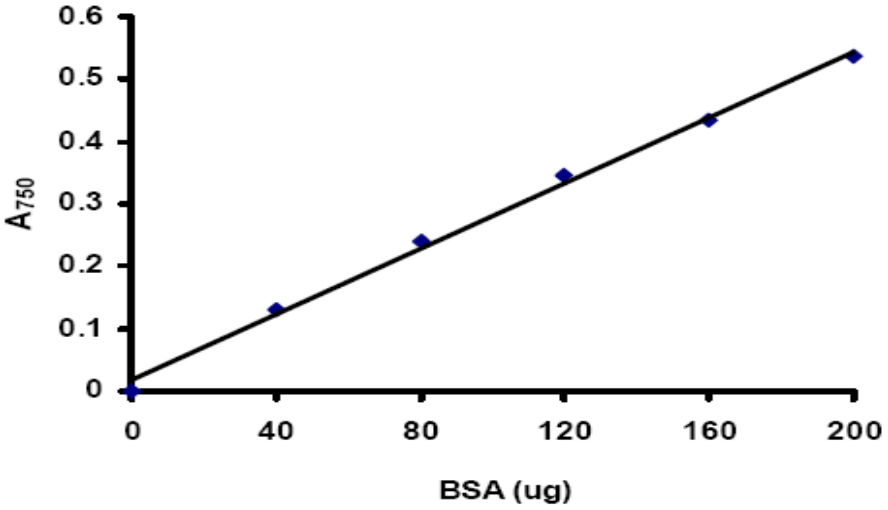
Graph 4.53 Effect of tributyrene on Ku-20



Partial purification of lipase

Partial purification of lipase was carried out by salting out with ammonium sulphate. The broth could not be precipitated beyond 80% properly. Protein estimation was performed by Foiln- Lawry's method (Graph- 4.54).

Graph 4.54 Standard Curve of Protein (BSA) by Folin-Lawry's Method



- Lipase from Mk-4 was purified 1.36 fold in 60%-80% ammonium sulphate fraction with 26.41 U/mg specific activity and 33.3% yield (Table-4.23)

Table-4.23 Partial purification of lipase from Mk-4

No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	3800	196	19.3	-	100
2.	**0-20%**	3	22	0.13	0.006	0.08
3.	**20-40%**	3	29.3	0.10	0.005	0.08
4.	**40-60%**	114	50	2.28	0.12	3
5	**60-80%**	1268	48	26.41	1.36	33.3
6	**80-100%**	180	8.3	21.68	1.12	4.7

❖ Partially purified lipase from Mk-18 was obtained in 60%-80% with 3.44 fold purification, 40 units/mg specific activity and 70% yield (Table-4.24)

Table-4.24 Partial purification of lipase from Mk-18

No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	2800	240	11.6	-	100
2.	**0-20%**	0	18	0	0	0
3.	**20-40%**	3	22	0.13	0.01	0.1
4.	**40-60%**	19	44	0.43	0.03	0.7
5	**60-80%**	1960	49	40	3.44	70
6	**80-100%**	211	11	19.18	1.65	7.5

❖ Lipase from Mk-23 was purified 1.98 fold in 60%-80% ammonium sulphate fraction with with 42.72U/mg specific activity and 42.2% yield (Table-4.25).

Table-4.25 Partial purification of lipase from Mk-23

No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	4760	220	21.63	-	100
2.	**0-20%**	10	9.8	1.02	0.05	0.2
3.	**20-40%**	20	12	1.67	0.08	0.4
4.	**40-60%**	23	43	0.53	0.02	0.5
5	**60-80%**	2008	47	42.72	1.98	42.2
6	**80-100%**	71	8.1	8.76	0.4	1.5

❖ Lipase from Ku-10 was purified 2.91 fold in 60%-80% ammonium sulphate fraction with with 29.1 U/mg specific activity and 55% yield (Table-4.26).

Table-4.26 Partial purification of lipase from Ku-10

No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	2060	206	10	-	100
2.	**0-20%**	11	21	0.51	0.05	0.53
3.	**20-40%**	10	21	0.5	0.05	0.4
4.	**40-60%**	20	38	0.52	0.05	1
5	**60-80%**	1134	39	29.1	2.91	55
6	**80-100%**	4.5	7.5	0.6	0.06	0.2

❖ Partially purified lipase was obtained from Ku-19 in the 40%-60% fraction with 5 fold purification, 57.76 units/mg specific activity and 54.15% yield (Table-4.27).

Table-4.27 Partial purification of lipase from Ku-19

No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	2240	194	11.54	-	100
2.	**0-20%**	12	19	0.63	0.05	0.5
3.	**20-40%**	30	23	1.3	0.1	1.3
4.	**40-60%**	1213	21	57.76	5	54.15
5	**60-80%**	90	25	3.6	0.3	4
6	**80-100%**	0	0.71	0	0	0

❖ Partially purified lipase was obtained from Ku-20 in 60%-80% fraction with 4 fold purification, 41 units/mg specific activity and 50.2% yield (Table-4.28).

Table-4.28 Partial purification of lipase from Ku-20

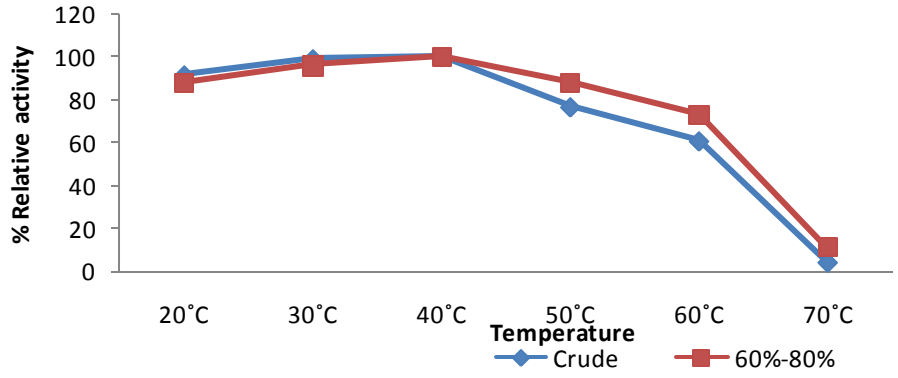
No.	Step	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (Units/mg)	Purification fold	Yield (%)
1.	**Crude enzyme**	1960	192	10.2	-	100
2.	**0-20%**	19	13	1.46	0.14	0.96
3.	**20-40%**	25	12	2.1	0.2	1.27
4.	**40-60%**	55	25	2.2	0.21	2.8
5	**60-80%**	983	24	41	4	50.2
6	**80-100%**	16	5.1	3.1	0.3	0.05

Parameters optimization for lipase activity

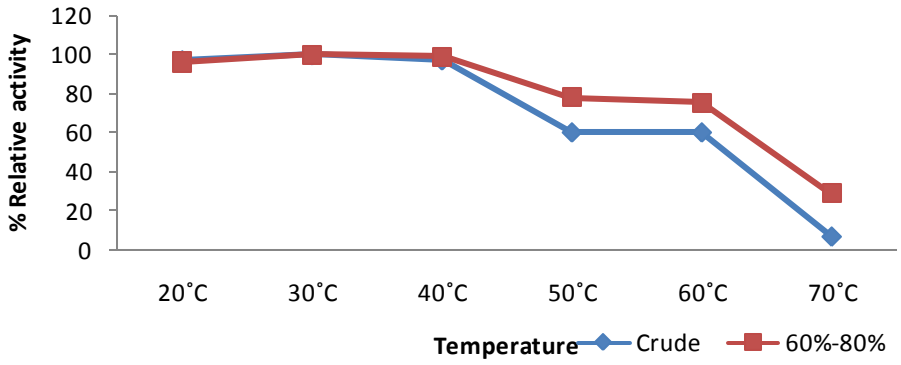
Crude and partially purified lipases were characterized for temperature optima, temperature stability, pH optima, effect of inorganic salt on activity etc.

❖ Optimum lipase activity from crude as well as partially purified enzyme samples from modrate halophilic isolate was observed in the range of 30-40˚C temperature. Optimum lipase activity from crude as well as partially purified enzyme samples from extreme halophilic isolate was observed in the range of 40-70˚C temperature. (Graph-4.55 to 4.60).

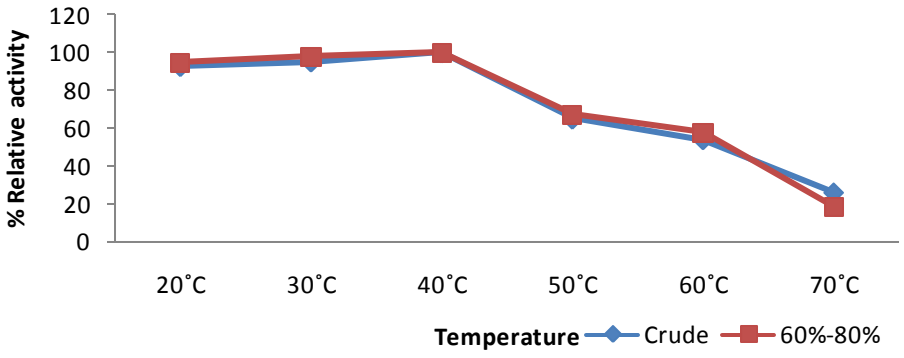
Graph 4.55 Temperature optima for lipase activity from Mk-4



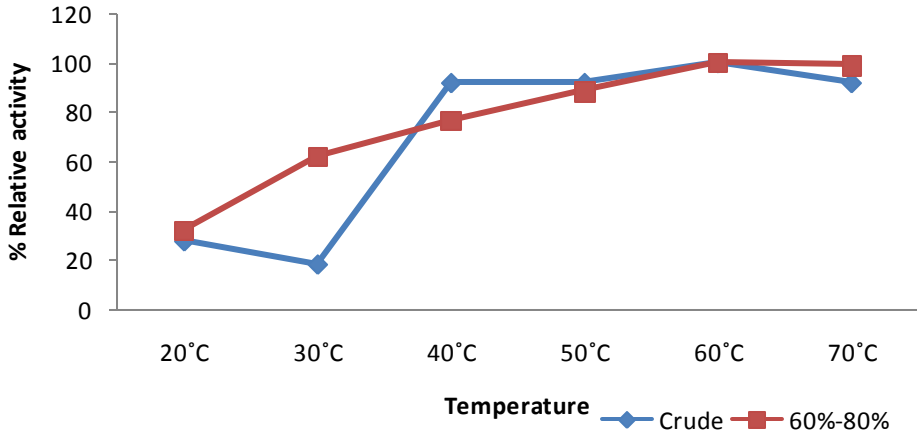
Graph 4.56 Temperature optima for lipase activity from Mk-18



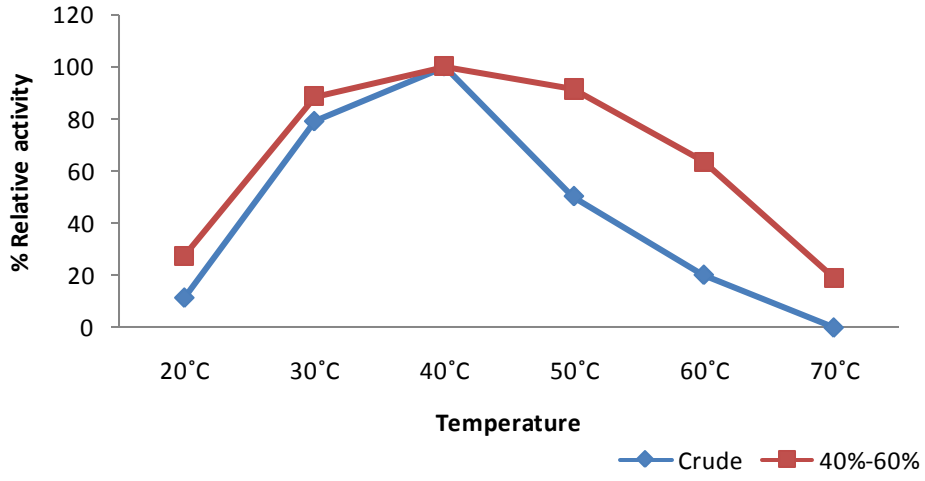
Graph 4.57 Temperature optima for lipase activity from Mk-23



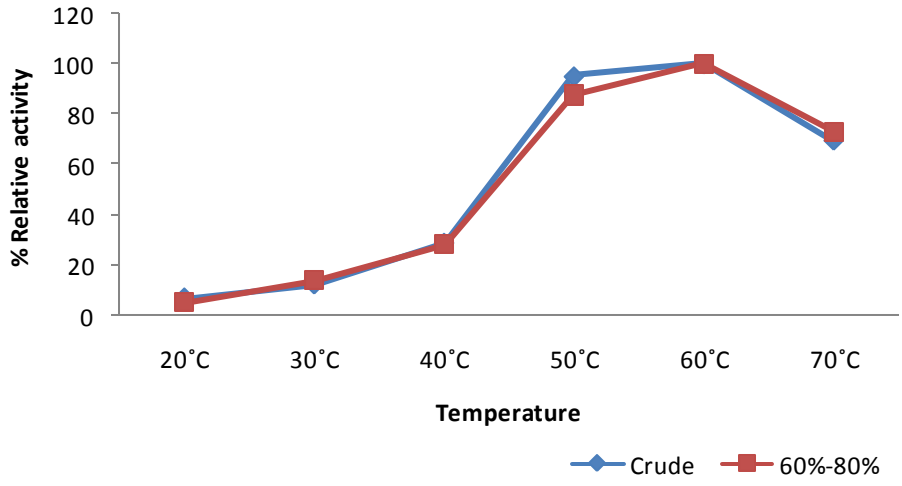
Graph 4.58 Temperature optima for lipase activity from Ku-10



Graph 4.59 Temperature optima for lipase activity from Ku-19

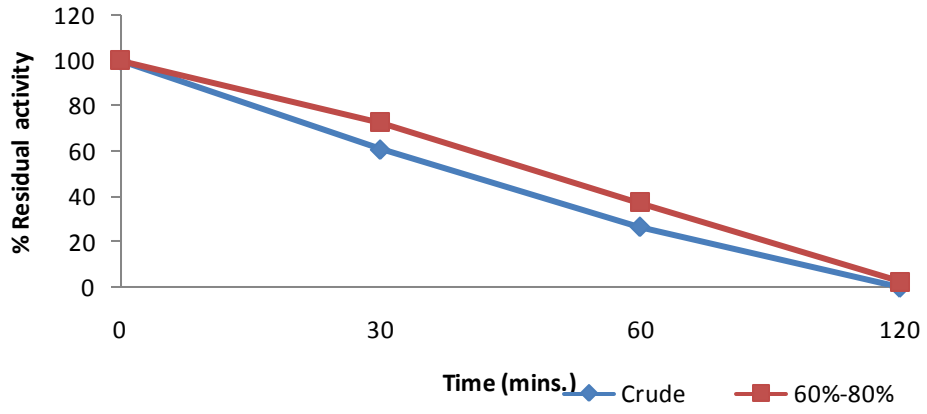


Graph 4.60 Temperature optima for lipase activity from Ku-20

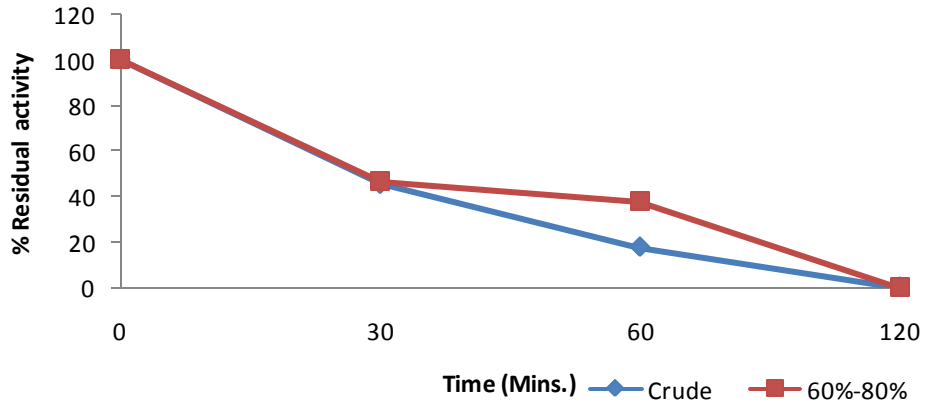


❖ The study of temperature stability from crude and partially purified enzymes from all the isolates was carried out at 60 and 70˚ C and both showed same pattern. Partially prified enzyme was found to be more stable as compare to crude sample (Graph-4.61-4.66)

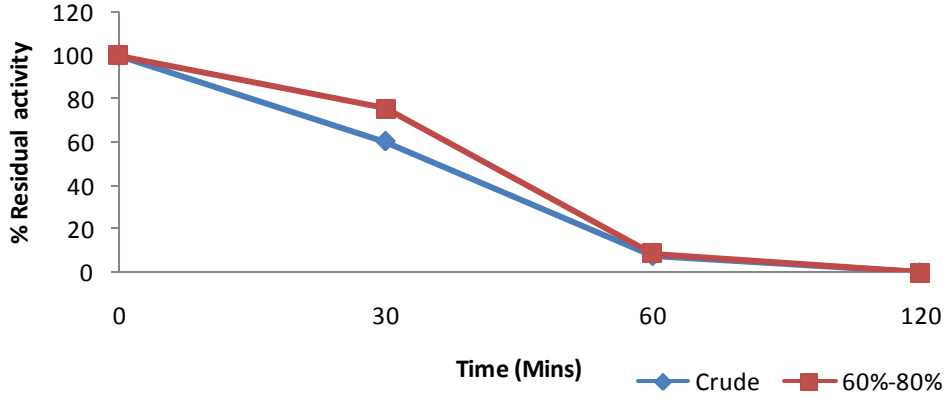
Graph 4.61 Temperature stability for lipase activity from Mk-4 at 60˚C



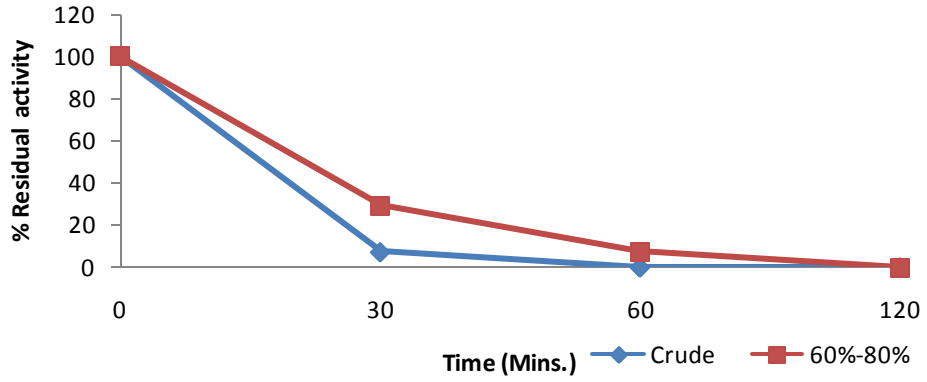
Graph 4.62 Temperature stability for lipase activity from Mk-4 at 70˚C



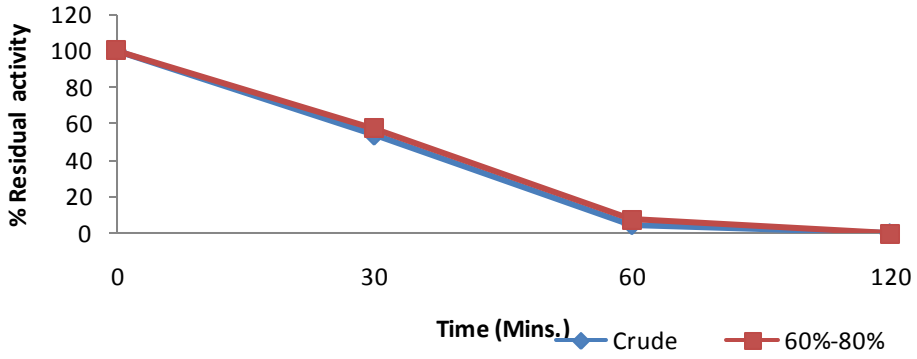
Graph 4.63 Temperature stability for lipase activity from Mk-18 at 60˚C



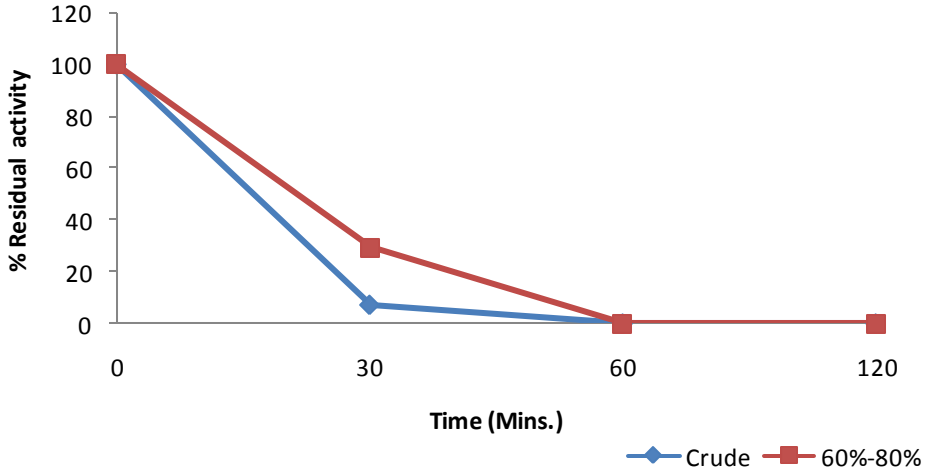
Graph 4.64 Temperature stability for lipase activity from Mk-18 at 70˚C



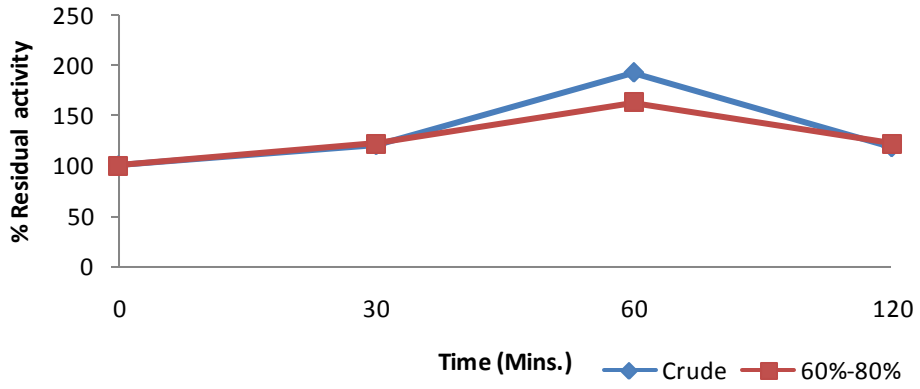
Graph 4.65Temperature stability for lipase activity from Mk-23 at 60˚C



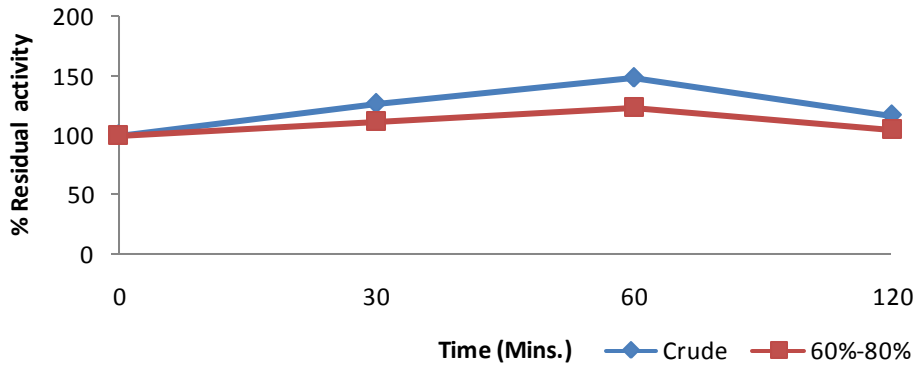
Graph 4.66 Temperature stability for lipase activity from Mk-23 at 70˚C



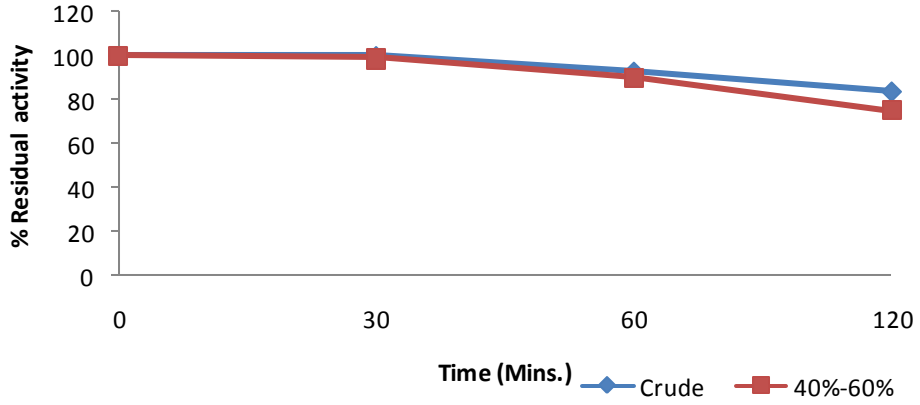
Graph 4.67Temperature stability for lipase activity from Ku-10 at 60˚C



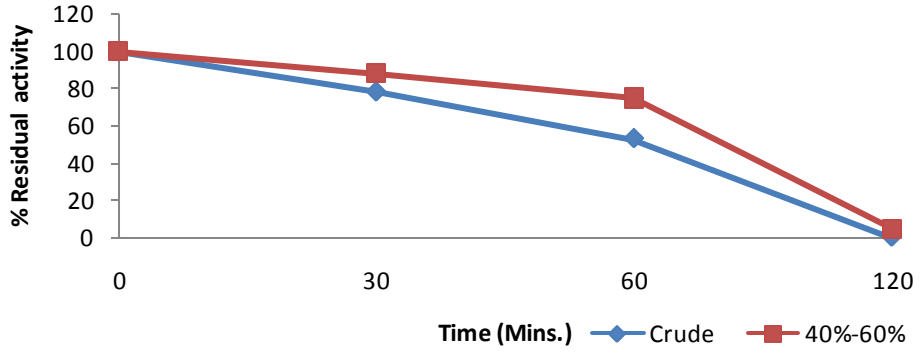
Graph 4.68 Temperature stability for lipase activity from Ku-10 at 70˚C



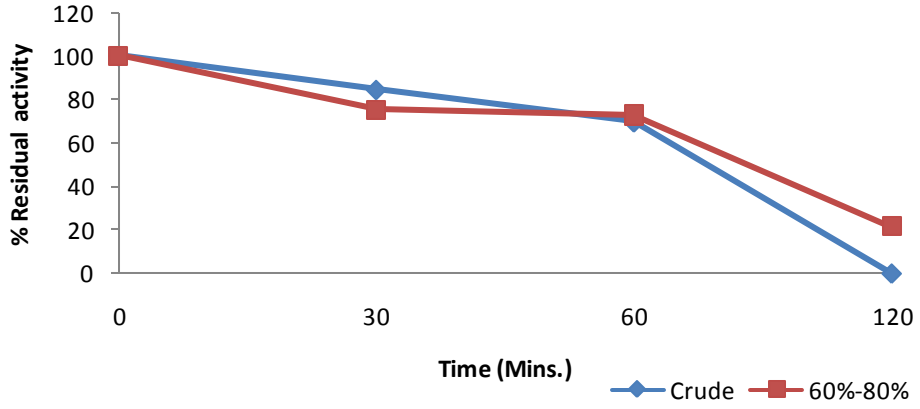
Graph 4.69Temperature stability for lipase activity from Ku-19 at 60˚C



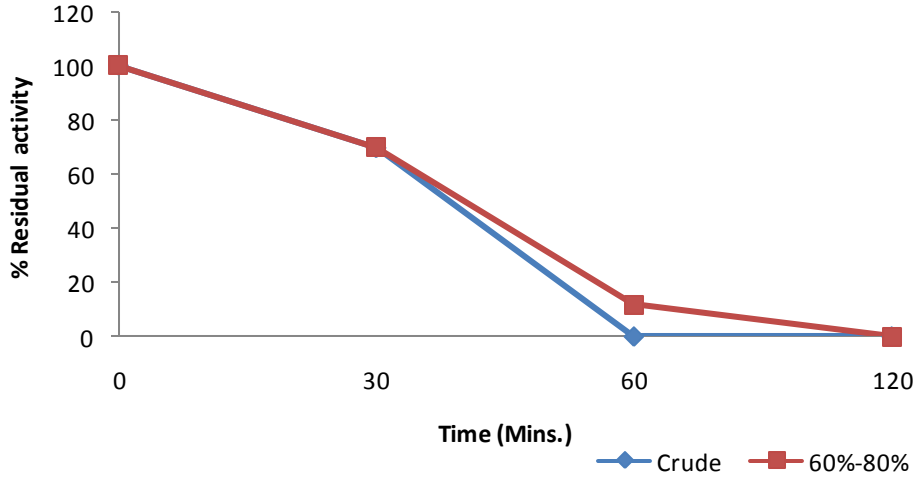
Graph 4.70 Temperature stability for lipase activity from Ku-19 at 70˚C



Graph 4.71 Temperature stability for lipase activity from Ku-20 at 60˚C

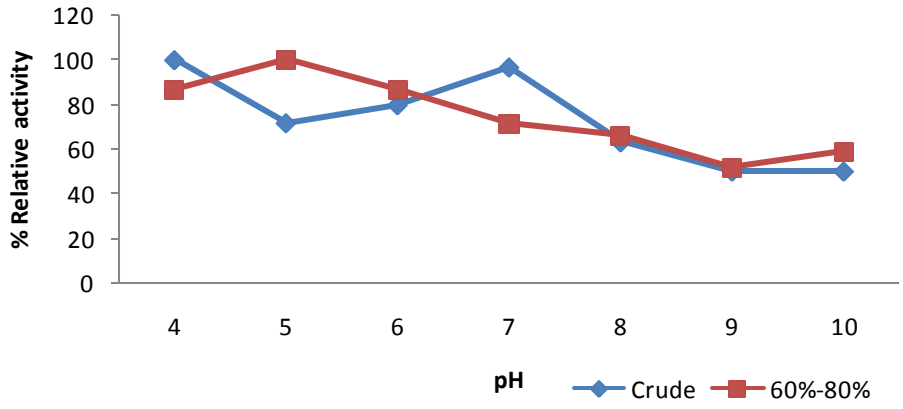


Graph 4.72 Temperature stability for lipase activity from Ku-20 at 70˚C

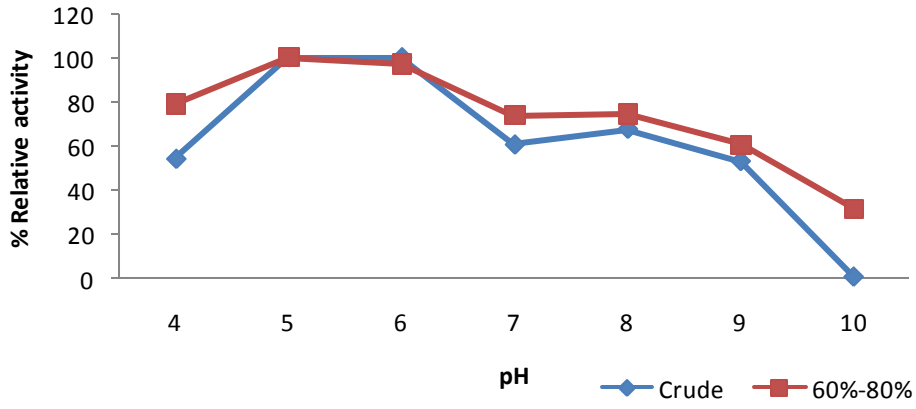


❖ Optimum lipase activity from crude as well as partially purified enzyme samples from modrate halophilic isolate was observed in the pH range of 4-6. Optimum lipase activity from crude as well as partially purified enzyme samples from extreme halophilic isolate was observed in the pH range of 6-8 (Graph-4.73 to 4.78).

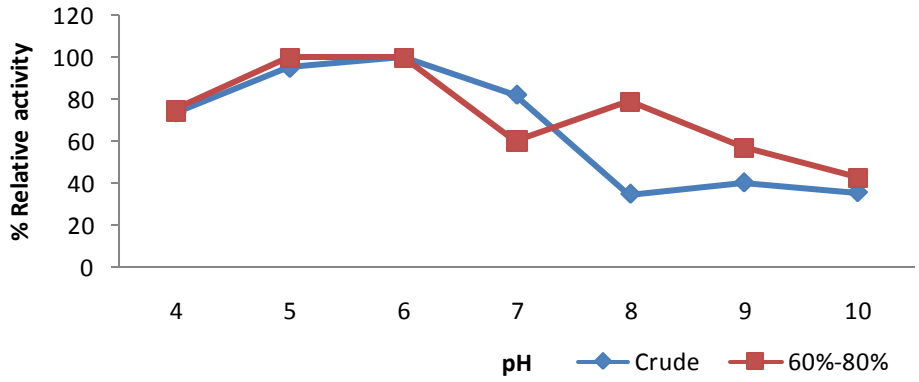
Graph 4.73 pH optima for lipase activity from Mk-4



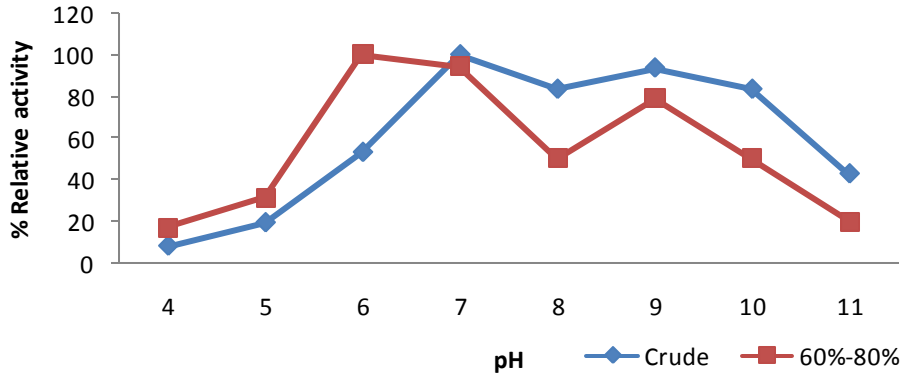
Graph 4.74 pH optima for lipase activity from Mk-18



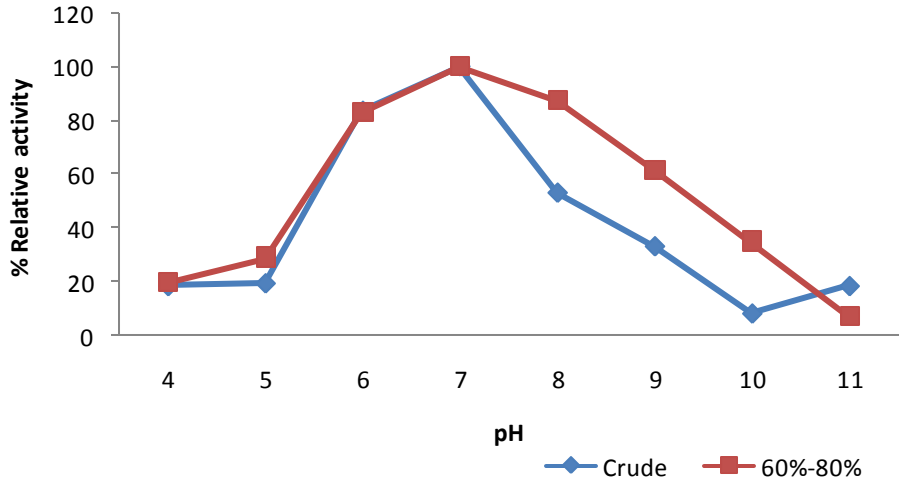
Graph 4.75 pH optima for lipase activity from Mk-23



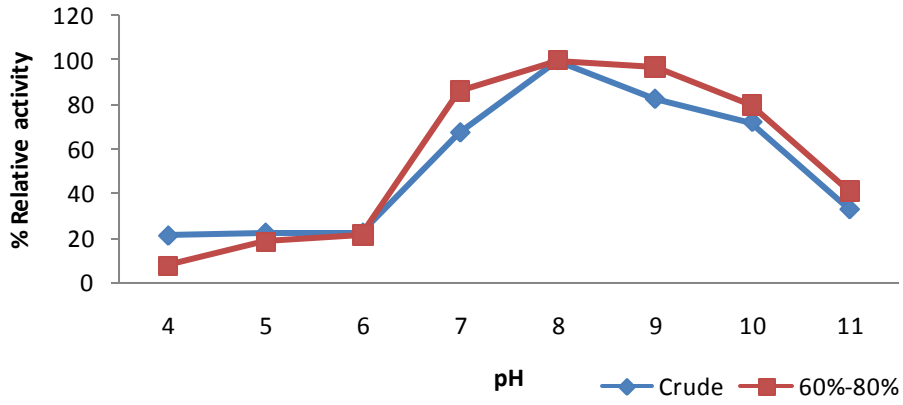
Graph 4.76 pH optima for lipase activity from Ku-10



Graph 4.77 pH optima for lipase activity from Ku-19

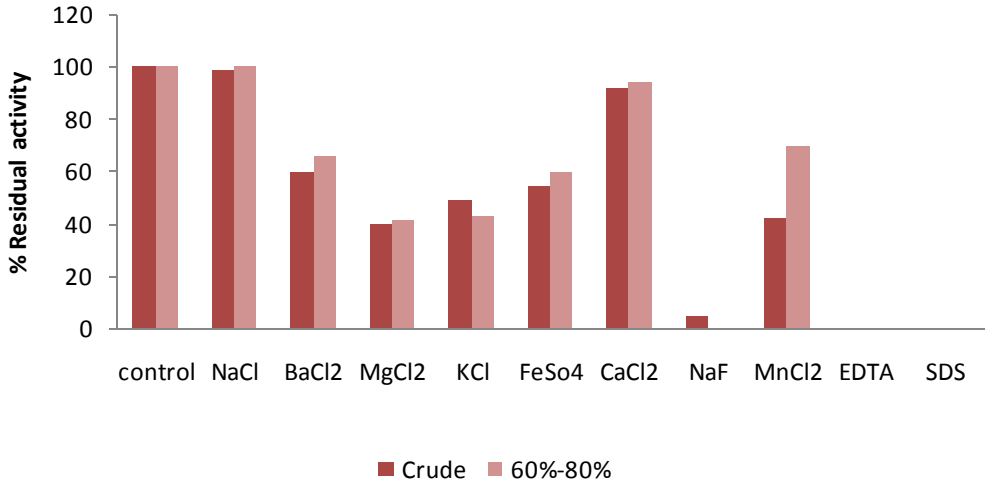


Graph 4.78 pH optima for lipase from Ku-20

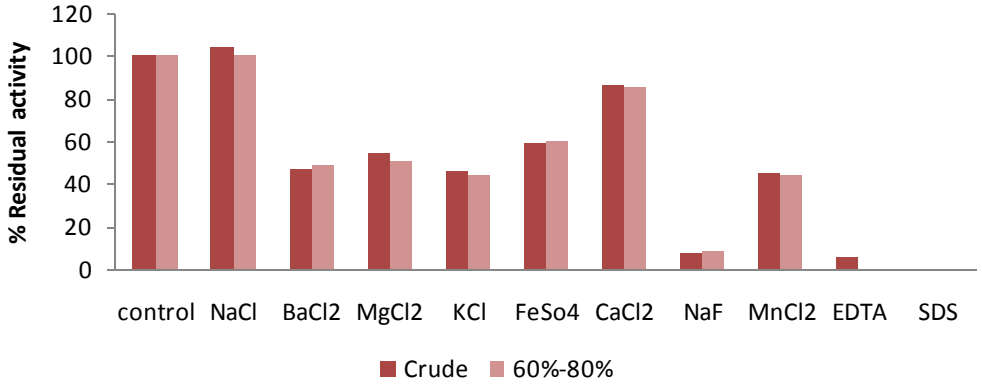


❖ Various inorganic salts were tested for their effect on lipase activity (Graph- 4.79 to 4.84). Crude and partially purified enzymes from moderate as well as extreme halophiles showed almost the same pattern of inhibition/induction by he salts.

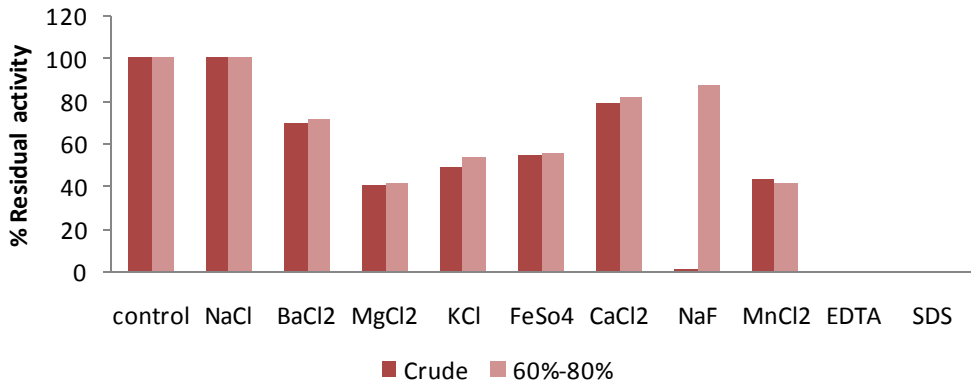
Graph 4.79 Effect of different inorganic salts on lipase activity from Mk-4



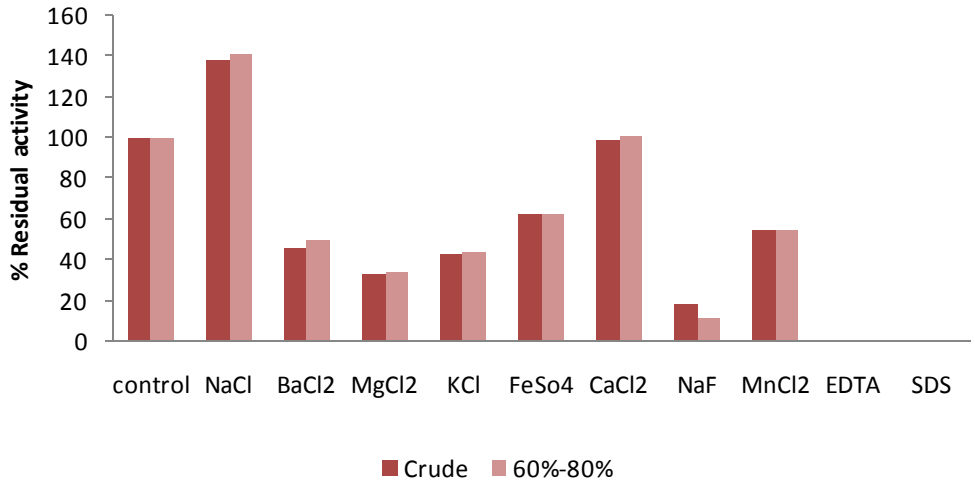
Graph 4.80 Effect of different inorganic salts on lipase activity from Mk-18



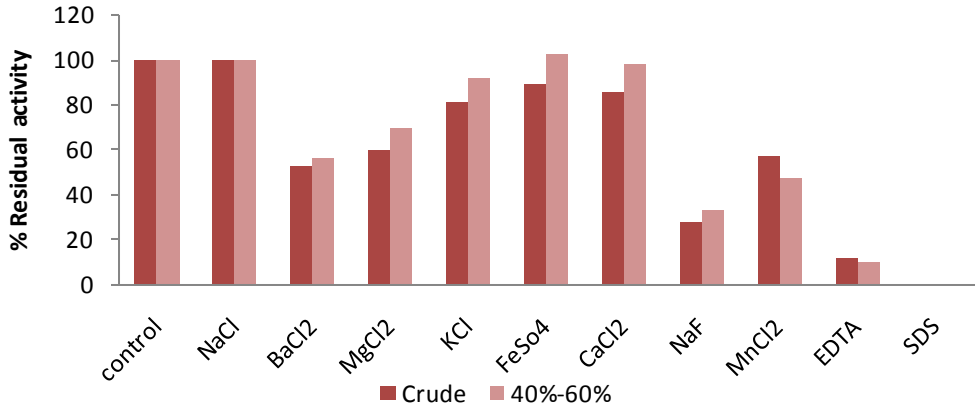
Graph 4.81 Effect of different inorganic salts on lipase activity from Mk-23



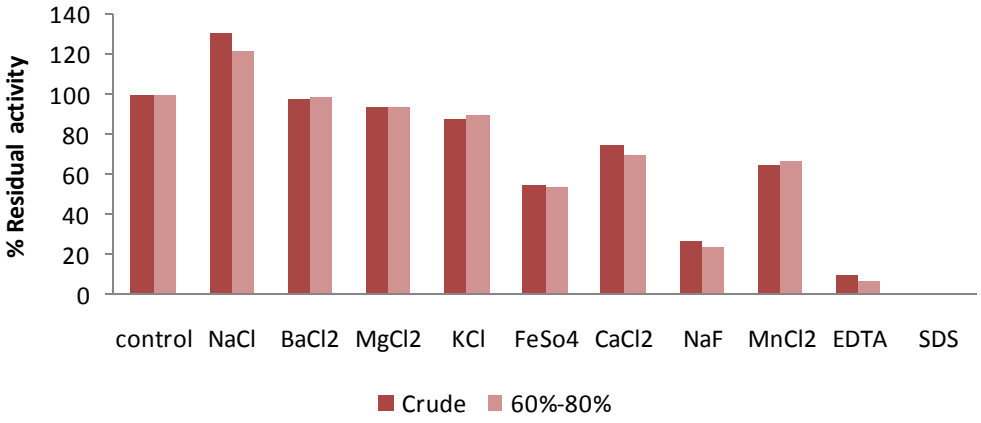
Graph 4.82 Effect of different inorganic salts on lipase activity from Ku-10



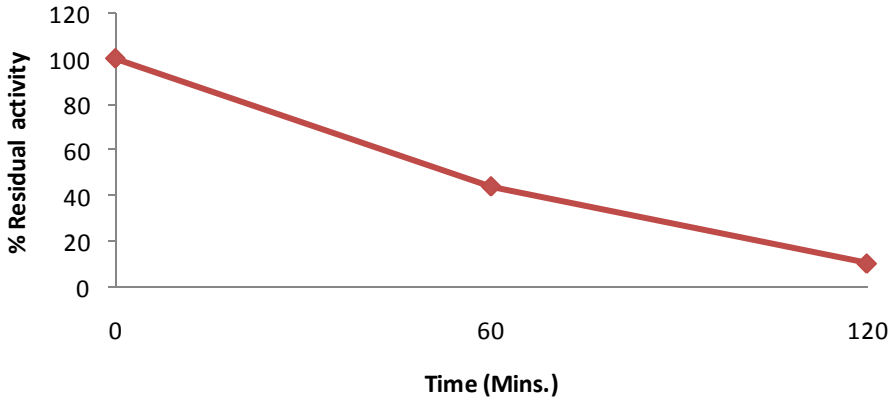
Graph 4.83 Effect of different inorganic salts on lipase activity from Ku-19



Graph 4.84 Effect of different inorganic salts on lipase activity from Ku-20

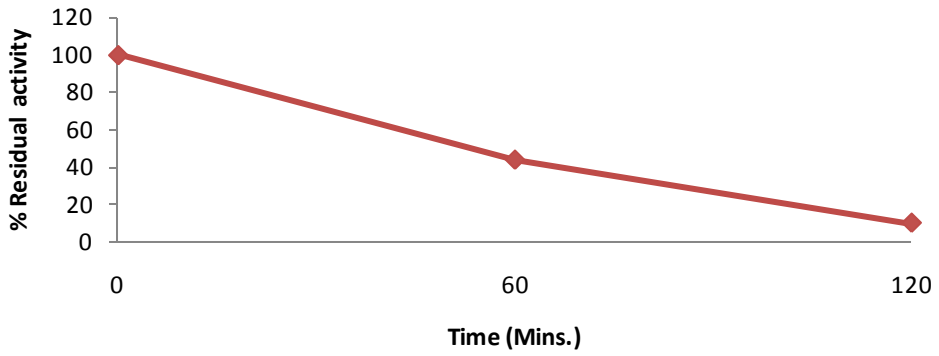


Graph 4.85 Effect of urea on lipase activity from Mk-4

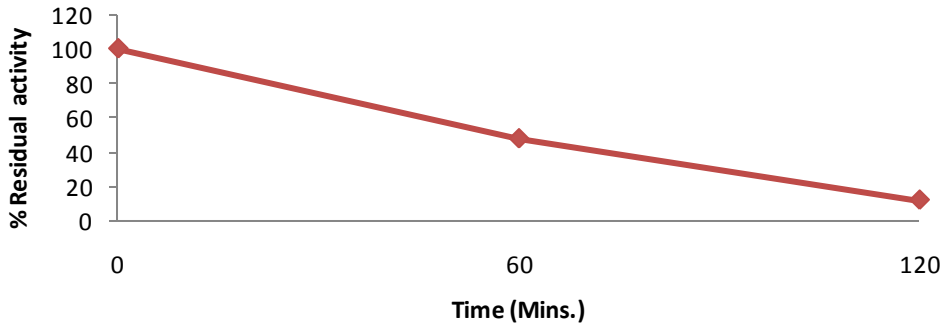


❖ Study of effect of 8M urea on activity of partially purified lipase from moderate and extreme halophilic isolate showed inhibitory effect as shown in graph- 4.86 to 8.91

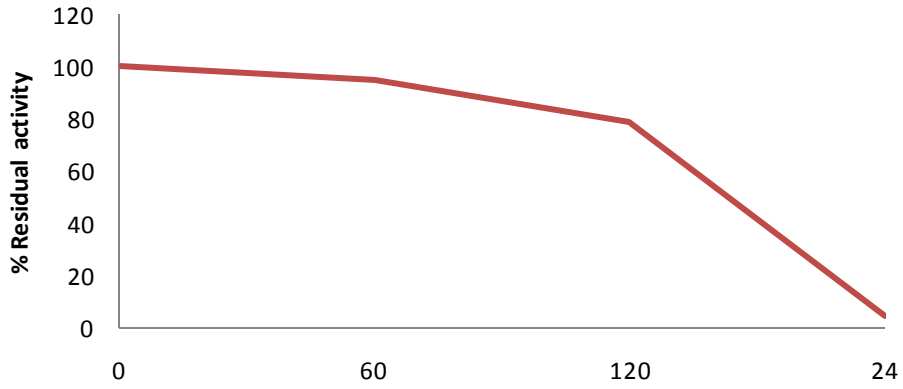
Graph 4.86 Effect of urea on lipase activity from Mk-18



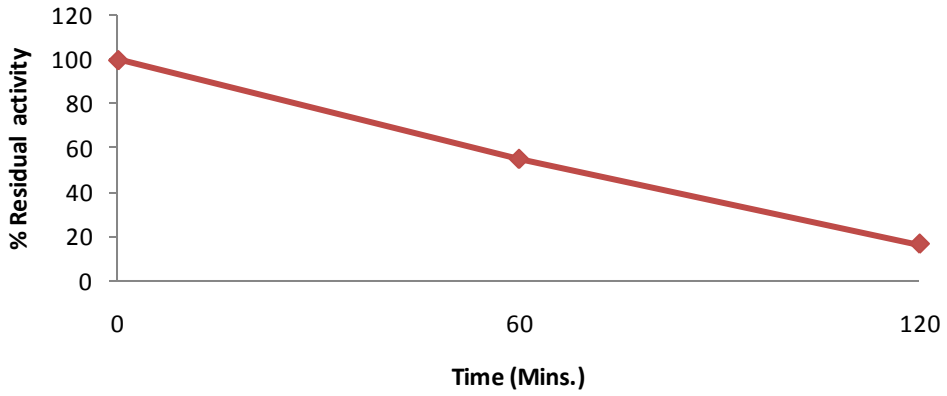
Graph 4.87 Effect of urea on lipase activity from Mk-23



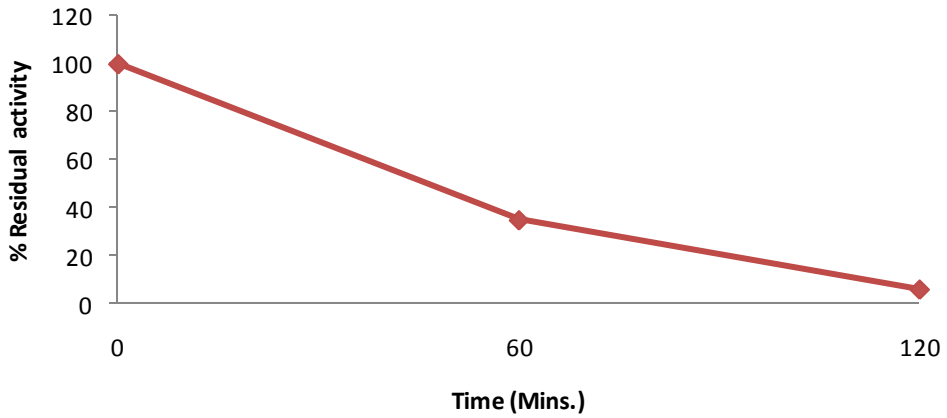
Graph 4.88 Effect of urea on lipase activity from Ku-10



Graph 4.89 Effect of urea on lipase activity from Ku-19



Graph 4.90 Effect of urea on lipase activity from Ku-20



- Study of effect of UV rays (As an environmental factor) was performed which indicated decline in population of halophiles as exposure time increased (at 280 n.m) as shown in table 4.29.

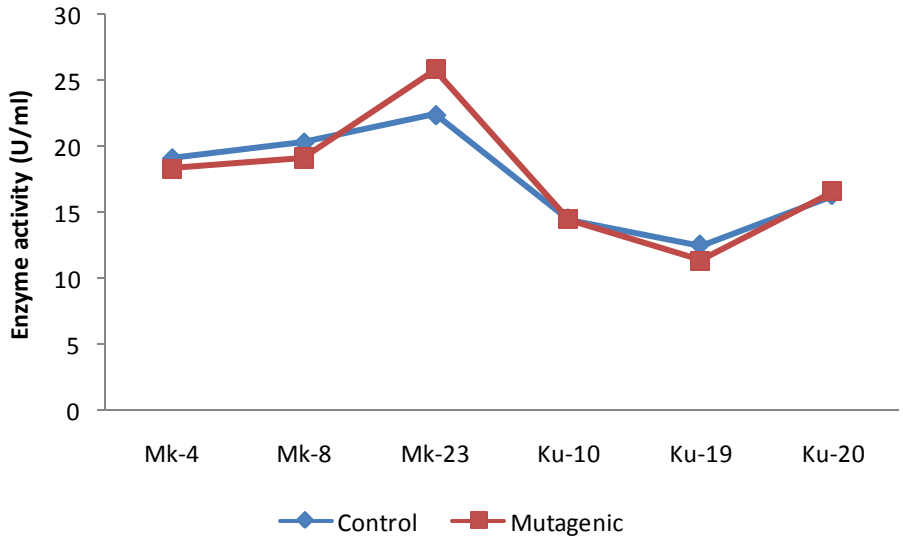
Table- 4.29 UV survival data of halophiles

Time (s)	Mk-4	Mk-18	Mk-23	Ku-10	Ku-19	Ku-20
0	25×10^{8}	20×10^{8}	1×10^{9}	14.7×10^{9}	88×10^{8}	33.6×10^{7}
10	60×10^{8}	12.3×10^{8}	11×10^{8}	……	……	55.1×10^{7}
20	98×10^{7}	11×10^{7}	1×10^{8}	19.2×10^{7}	23.8×10^{7}	10.4×10^{7}
30	22×10^{7}	12.8×10^{7}	27×10^{7}	61.9×10^{5}	35×10^{6}	7.8×10^{5}
40	10×10^{6}	……	2.5×10^{7}	11.2×10^{5}	31.55×10^{4}	12.8×10^{5}
50	17×10^{5}	……	39×10^{6}	51×10^{4}	34.9×10^{4}	12.2×10^{5}
60	20×10^{4}	……	21.9×10^{6}	31.5×10^{4}	18.8×10^{4}	……
70	19.8×10^{5}	14.2×10^{4}	23×10^{5}	21.5×10^{4}	21.65×10^{3}	34×10^{4}
80	21.8×10^{4}	75×10^{3}	20×10^{4}		36.25×10^{3}	……
90	0	31.5×10^{3}	22.8×10^{5}	17.8×10^{4}	……	……
100	0	22.8×10^{3}	11×10^{3}	23×10^{1}	……	28×10^{2}
110	18.9×10^{3}	40×10^{3}	99×10^{2}	326	220	……
120	50×10^{2}	65×10^{2}	31×10^{2}	164	143	5×10^{2}

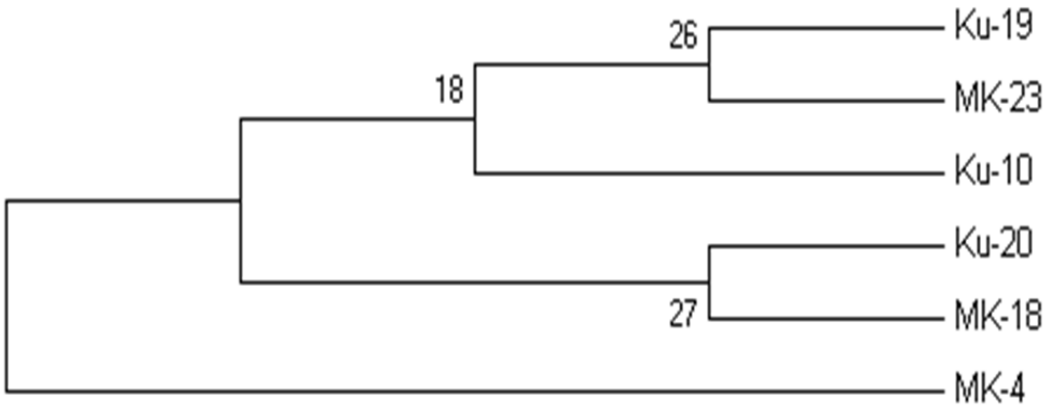
UV mutagenesis for lipase

All the six isolates were exposed in UV radiation in order to carryout random mutagenesis in lipase producing gene for the improvement of lipase production. From moderate halophiles, isolate Mk-23 showed 3.4 U/ml increase in lipase production after UV exposure. Other five isolates did not show any considerable increase in lipase production (Graph-4.91).

Graph 4.91 Effect of UV irradiation on lipase production mutagenesis



- Phylogenetic analysis on the basis of 16's r-RNA sequence analysis was carried out and dandogram was prepared to study phylogeny among isolates.



CHAPTER-5

Discussion

- Till date, most of the research on halophilic organisms has been done from halophiles isolated from soda lakes, Dead Sea, Sea water etc. Present research is on the unique halophilic bacteria in coastal region of Gujarat, particularly from Little *Rann* of Kutch and intestine of wild ass living in the saline desert of Kutch. India is having a great diversity of halophiles as it contains two oceans, two gulfs and a bay as well as saline deserts.

- With this background, total 54 salt tolerant organisms were isolated from Little *Rann* of Kutch and its nearby areas. 30 extreme halophiles were isolated from samples collected from back water of sea, mud and salt samples while 24 moderate halophiles were isolated from excreta of wild ass, collected from wild ass sanctuary in this area. The samples vary vastly in their physical appearance as well as chemical, moisture and microbial content indicating existing of different ecological niche in the same geographical as well as climatic region.

- Enrichment of halophilic organism is essential for their isolation from natural samples. Enrichment can be performed on various media containing varying and gradually increasing NaCl concentrations. Organisms isolated from sea water, salt and mud samples were able to tolerate salt concentration up to saturation point in the media formulation indicative of their extreme halophilic nature while organisms from excreta of wild ass were growing in presence of NaCl concentrations up to 15%. No growth was observed at salt concentrations below 5% indicative of halophilic nature of organisms. Presence of moderate halophiles in excreta of wild ass is an indication of presence of same organisms in their intestine. This is very unusual and suggests that the normal intestinal flora of these animals have been replaced by halophiles. It was also observed that all 24 moderately halophilic isolates were able to tolerate bile salt which is a true indicative of their intestinal origin. The presence of such halophiles in the intestine of these animals as their normal flora could be due to presence of significant amount of salt in local grass and as wild ass is herbivore, its intestinal microbes may have adapted, got eliminated and/or replaced and have formed present moderate halophilic community.

- Colony characterization of extreme as well as moderate halophiles indicates that they are highly diversified. Different isolates were able to form colonies of different size, shape, color, texture, margin, elevation etc. Morphological studies by staining indicate that all the organisms were Gram positive or Gram variable, medium or long rods. All the moderate halophiles isolated from excreta of wild ass were Gram positive long rods. Many literature survey shows that majority of moderate halophiles studied in detail till date belongs to Gram negative group and information regarding Gram positive moderate halophiles is rare (Ventosa *et al.*, 1998). Present studies are focused on the characterization of Gram positive moderate halophiles. Extreme halophiles were also able to produce reddish color pigments indicative of their true extreme haloarcheal origin. Haloarchaea are able to produce red color pigments carotenoids (C_{50}) known as bacterioruberins (Grant and Larsen, 1989).

- Bergey's Manual of Systemic Bacteriology is useful manual for biochemical identification of Bacteria. On the basis of biochemical tests performed, it was observed that few isolates were able to give Methyl Red test and Voges-Proskaur test positive, suggesting their ability to produce acid from glucose and also of their chemotropic nature. Majority of isolates were positive for catalase test which indicate aerobic nature of these organisms. Mancinelli and Hochstein in 1986 described that mainly *Haloarcula* and *Haloferax* genera of halophiles are able to reduce nitrate to nitrite. Majority of our isolates were able to give nitrate to nitrite test positive and hence indicates similarities with above two listed genera. Halophilic isolates were also diversified in terms of sugar utilization like glucose, sucrose, fructose, maltose, xylose etc. Growth at 50˚C in case of moderate halophiles was found positive suggesting their ability to withstand higher temperature during extreme summer in such geographic zones. Ability to utilize glucose by both moderate and extreme halophiles and their ability to produce catalase suggest their evolutionary relationship. Extreme halophiles were found to be susceptible to bile indicating their non-intestinal origin. In ability to grow on MacConkey's and EMB agar suggest their Gram's positive nature.
- Halophiles are valuable for enzyme production commercially. Data indicates that many halophiles are able to produce industrially important enzymes like lipase,

amylase, protease, cellulase and Chitinase on media containing specific substrate and can be screened on the basis of development of zone of substrate utilization. All the isolates were screened for production of extracellular enzymes amylase, lipase, protease, cellulase and Chitinase. Some of the moderate halophiles were able to produce amylase, lipase as well as protease while very few could produce Chitinase and cellulase. Interestingly, the moderate halophilic isolates Mk-9 which could produce significant quantity of cellulase and Chitinase could not produce protease or lipase but was amylase positive. This indicates that this isolate preferred carbohydrate compared to proteins and lipids and hence is a novel organism.

- Lipase production was detected on media containing tributyrene and olive oil as a substrate. Werasit and Anan, 2007 have screened lipase producing organisms in the medium containing 2% Tween-80 and 1-4 M NaCl. Positive organisms were detected on the basis of zone of substrate utilization surrounding colony. Primary screening of lipase producing organisms can be performed on medium containing 0.01% CaCl2 and Tween 80 (Gonzalez *et al.*, 1978). Even lipase producer can be screened on basal MH medium supplemented with 2.5% olive oil (w/v) and 0,001% rhodamine B Positive organisms can be identified by the presence of an orange-red halo under UV light (Bhatnagar *et al.*, 2005). Amylase producing organisms was screened on medium containing starch and positive organisms were detected on the basis of clear zone surrounding colony after adding iodine solution. Gonzales *et al.*, 1978 have screened amylase producing organisms on medium containing 2 gm/lit starch and positive strains were screened on the basis of clear zone surrounding colony after adding I_2-KI solution (0,1% I_2 – 0,2% KI). Third most important enzyme, protease was screened on medium containing casein or gelatin as proteinic source. However, Ventosa, 1982 have screened proteolytic halophiles on saline medium containing 50% Milk. Cellulase producing organisms were screened on Dubo's medium containing Carboxymethyl cellulose and Chitinase producing organisms were screened on chitin agar medium.

- In case of extreme halophiles lipase production was found positive in all the isolates but protease and amylase was produced only by few isolates. Isolate Ku-8,

Ku-12, Ku-14, Ku-16, Ku-20 and Ku-27 were able to make amylase, protease and lipase in almost in same amount which indicate its diversified metabolic diversity as compare to other isolates.

- Isolates were variable in terms of growth at different temperature and pH. Most of the isolated organisms were unable to utilize urea and tryptophan. The data indicates absence of enzymes urease and tryptophanase. Similar types of results were also obtained by various investigators (Muntyan *et al.*, 2002; Romano *et al.*, 2005).
- Enzyme production by moderate halophiles on solid media was found to be affected by pH, NaCl concentration and temperature. These organisms have been found to produce amylase in the pH range of 5-7 with some isolates having even pH 4 as a significant (isolate Mk-9, Mk-15), 10-11% NaCl concentration and at 30˚C temperature.
- Amylases catalyse the cleavage of the *a*-1, 4 linkage of starch, yielding short linear maltodextrins, have many commercial applications, particularly in the food and detergent industries. The use of amylases from halophilic bacteria in industrial processes would have the advantage of the enzymes having optimal activities at high salt concentrations (Kamekura, 1986; Ventosa and Nieto, 1995). Amylase production and media optimization in liquid media was performed for most efficient amylase producer moderate halophiles i.e. Mk-21. Growth kinetics with reference to amylase production from Mk-21 shows that maximum amylase production was after 72 hours (0.7 U/ml) and then gradually declines. Amylase production starts at the initiation of stationary phase and became maximum at mid stationary phase. Comparative higher amylase producer *Halobacillus* sp. strain MA-2 was able to produce 3.2 U/ml amylase (Amoozegar *et al.*, 2003). Amylase production affected by pH, Mk-21 was able to produce highest lipase at slightly acidic pH (5, 6) and mesophilic temperature. The data are compatible with amylase from *H. meridiana* (Coronado *et al.*, 2000), while not compatible with *Bacillus* sp. Strain *TSCVKK* (Kiran *et al.*, 2001). The organism was able to produce maximum amylase at 1.2% starch (w/v), compatible with amylase from *Micrococcus halibius* sp. ATCC 21727 (Shapiro and Lionel, 1971). The study of effect of nitrogen source on amylase production from Mk-21 was carried out showing peptone to be the most preferred or efficient nitrogen source.

- The data indicates that area near sea shore may be contaminated by oily waste. Then media and environmental parameters were optimized for maximum lipase secretion on solid media. Optimum pH was found to be 4-6, NaCl concentration 10-11%, w/v, and temperature in the range of 30-40˚C for moderate halophiles. Effect of tributyrene concentration was positive from 1-4% v/v but was inhibitory from 5% onwards suggesting that tributyrene may exert suppression of its own utilization. Study of effect of pH on lipase production by extreme halophiles on solid media shows a wide range of pH from 4-10. This indicates a broad range of pH stability of this enzyme in extreme halophiles. This is also in accordance with varied pollutants and wastes entering into sea water or nearby area and causing drastic shift in pH. This may also indicates adaptive nature of the organism with reference to lipase production and these characteristics can be exploited industrially for the production of lipase capable of activity in wide pH range. These organisms could grow at 10-35% NaCl concentration, were able to produce considerable amount of lipase at this NaCl concentration but maximum lipase was found to be produced from 10-15% NaCl concentration range. These aspects can also be used industrially for the production of salt tolerant lipase. The temperature at which these organisms produces maximum lipase was 30-40˚C while some could grow at 60˚C also indicating stability of this enzyme at 60˚C temperature.
- On the basis of zone index, 3 organisms from moderate halophiles viz. Mk-4, Mk-18, Mk-23 and 3 organisms from extreme halophiles viz. Ku-10, Ku-19, Ku-20 were selected for further studies on liquid media.
- When selected halophiles were grown in liquid media, all the organism were found to produce maximum lipase in early, mid or late stationary phase. Above results indicate the role of lipase in ecological adaptation of halophiles in nature when primary nutrient get exhausted. Highest extracellular lipase activity from Mk-4, Mk-18 and Mk-23 were 20.9 U/ml, 21.2 U/ml and 22.4 U/ml respectively after 264 hours. Above discussion indicates that Mk-23 is the most efficient lipase producer in liquid media. In case of extreme halophiles maximum lipase production in terms of U/ml was 12.1 U/ml, 13.5 U/ml and 12.3 U/ml from Ku-10, Ku-19 and Ku-20 respectively after 264 or 288 hour. This indicates that extreme halophile requires longer time for lipase production as compare to moderate halophiles. This could be due to high salt concentration interfering in

enzyme synthesis, activity or enzyme integrity. Isolate Ku-20 was found to produce maximum lipase in shortest time (192 hours) amongst all extreme halophiles but the activity decreased drastically at 264 hours. Similar types of results were obtained by Werasit and Anan, 2007 from halophilic *Staphylococcus warneri* PB233 at a stationary phase. The maximum production of lipase was 90.12 U/ml at 48 hr.

- Media optimization at industrial scale is necessary in order to obtain maximum yield. Media optimization was performed by classical method in which one independent variable was changed and remaining all other variables were constant at a time. Optimization of pH for halophilic lipase production states that moderate halophiles able to produce maximum lipase at acidic to slightly alkaline (6-8) pH. Extreme halophile prefers neutral to alkaline (7-9) pH. This indicates that lipase from moderate halophiles isolated from excreta of wild ass is exposed to wide pH variation in animal intestine and hence posses wide pH range for production and activity of lipase. Extreme halophiles on the other hand have slightly alkaline pH in their habitat (pH-8) and hence prefer such pH for lipase activity. Similar types of results were obtained in case of marine *salinivibrio* SA2 (Amoozegar *et al*., 2008) and *pseudomonas* (Yapasan, 2008) but slightly lower than *Marinobacter hydrocarbonoclasticus* AK5 (Anilkumar *et al*., 2010). In case of moderate halophile Mk-23 lipase activity was found to be maximum when the cell growth was at its minimum (pH-6-7) again suggesting that utilization of lipids is not the primary mode of metabolism of these organisms.
- Lipase production in liquid media was also found affected by NaCl concentration. Maximum lipase production in case of moderate halophiles was found at 10-11% NaCl concentration while for extreme halophiles it was 10-15%. This suggests that lipase produced by moderate as well as extreme halophiles have different stability at different NaCl concentrations and this property can also be exploited at industrial level.
- Most of moderate halophiles and extreme halophiles prefer to produce maximum lipase at 30˚C-40˚C. The data indicates that both moderate and extreme halophiles able to produce maximum lipase in mesophilic temperature range. Similar types of results were also obtained from other halophiles isolated from subterranean rock

salt crystal (Roxana *et al*., 2009), *pseudomonas* (Yapasan, 2008) and *Marinobacter hydrocarbonoclasticus* AK5 (Anilkumar *et al*., 2010).

- The optimum Tributyrene concentration for lipase production was found to be 4-6% v/v and 1-6% v/v for extreme halophiles. This can be due to variation in the availability of lipoidic materials in sea water or salt samples from where extreme halophiles have been isolated. Further addition of tributyrene decreased the biomass production drastically suggesting catabolic repression or end product repression of the metabolic pathway that utilizes tributyrene.
- Purification of halophilic lipase is important at larger scale and for commercial applications. Purification of enzymes can be performed by various strategies like Salt precipitation, gel filtration chromatography, ion exchange chromatography, hydrophobic interaction chromatography, reversed phase HPLC, affinity chromatography, organic solvent extraction etc. (Ali *et al*., 2010). Purification techniques should be able to give high purity, easier to be operated at larger scale and should be reproducible. Among all above techniques, salt precipitation by ammonium sulfate fractionation is easier and inexpensive technique. This technique is having the advantage that most of the precipitated enzymes are not permanently denatured can be redissolved with restoration of activity. The basis of fractionation in this method is that, as the salt concentration of the extract is increased, proteins with larger or more abundant hydrophobic patches will precipitate before those with smaller or fewer patches and protein would precipitate. All the halophilic isolates were able to produce extracellular lipase which was fractioned by adding ammonium sulfate and enzyme activities as well as protein content were determined. Lipase from Mk-4 was purified 1.36 fold purification in 60%-80% ammonium sulphate fraction with 26.41 U/mg specific activity and 33.3% yield. Above data is much lower than lipase from *F. oxysporium*, lipase from this organism is found to give 18.1 U/mg specific activity, 4.3 fold purification and 84.7% yield in 60%-90% fraction (Hala Mohamed *et al*., 2010). Lipase from Mk-18 was obtained in 60%-80% fraction with 3.44 fold purification, 40 units/mg specific activity and 70% yield. Lipase from *Thermosyntropha lipolytica* shows higher purification fold as compare to Mk-18 but lower specific activity and % yield (Mohemad *et al*., 2007). Lipase from Mk-23 shows intermediate purification fold and yield to that of Mk-4 and

Mk-18 and shows purification fold 1.98 in 60%-80% ammonium sulphate fraction with 42.72 U/mg specific activity and 42.2% yield.

- Lipase from Ku-10 shows slight higher purified enzyme as compare with Mk-23 while lower to Mk-18 in 60%-80% fraction with 2.91 purification fold, 29.1 U/mg specific activity and 55% yield. Above data are non compatible with lipase from *pseudomonas* (Kanwar *et al*., 2002) Lipase from Ku-19 shows highest purification among all isolates and purified fraction of lipase having 5 fold purification, 57.76 units/mg specific activity and 54.15% yield. Ku-20 shows lower lipase yield as compare to Ku-19 but still higher yield. Purified lipase fraction from Ku-19 shows 4 fold purification, 41 units/mg specific activity and 50.2% yield.
- It is also important in enzymology to characterize enzyme for knowing its important properties to cope with industrial applications. pH is important factor that affects lipase activity and stability. pH optima for crude and partially purified enzymes were checked by incubating enzyme-substrate mixture at different pH followed by measurement of enzyme activity. Lipases from moderate halophiles were found to most active in acidic pH and activity gradually declines as pH shifts towards alkaline side. It might be due to the fact that changes in the external pH optima may also alter the ionization of the nutrient molecules and thus, reduced their availability to the organism. This data again indicates intestinal origin of moderate halophiles. The results are comparable with lipase from *Aspergillus* sp. Active in the acidic range of pH (Cihangir and Sarikaya, 2004) while above data are not comparable with lipase from *Geotrichum marinum* (Huang *et al*., 2004) and *Rhizopus oryzae* (Salah *et al*., 2006) whose lipase active maximally in alkaline pH. Lipases from extreme halophiles were most active in alkaline pH and activity diminished in acidic side. These results are compatible with extracellular lipase from *Staphylococcus warneri* (Werasit and Anan, 2007), *R.oligosporus var. microspores* (Tehreema *et al*., 2011) and Marine *Vibrio fischeri* (Ranjitha *et al*., 2009) while slightly lower than *p. aeruginosa* whose extracellular lipase have maximum activity at pH 9 (Hesham *et al*., 2005).
- Temperature generally affects enzyme activity and stability. Higher temperature causes denaturation of protein while lower temperature has static effect and hence used for preservation. Crude and partially purified lipase from Mk-4 shows maximum activity at 40˚C and became unstable at 70˚C while shows less

temperature stability at 60˚C and 70˚C after 60 min. and became inactivated after 120 min. temperature optima and temperature stability data are comparable with lipase from halotolerant *Staphylococcus warneri* (Werasit and Anan, 2007). Crude and partially purified lipase from Mk-18 shows similar temperature optima and temperature stability with lipase from Mk-4, but less stability at 70˚C. Mk-23 lipase shows even less heat tolerance as compare to Mk-4 and Mk-18, highest activity at 40˚C while became inactive at 60˚C and 70˚C after 60 mins. Temperature optima and temperature stability data of lipase from moderate halophiles are similar to temperature tolerance of lipase from mutant *R. oligosporus* (Tehreema *et al*., 2011), *Penicillium* sp., (Maliszewska and Mastelerz, 1992) and *R. oryzae* (Hiol, 2000).

- Lipase from Ku-10 shows highest temperature tolerance among all isolates and shows highest activity at 70˚C and more than control data after incubation at 60˚C and 70˚C even after 60 and 120 min. of incubation. Such type of thermostable lipase was obtained from *Salinivibrio* sp. strain SA-2, which
- retains 90% of its activity at 80 ℃ for 30 min (Amoozegar *et al*., 2008). Extracellular lipase from Ku-19 and Ku-20 shows highest activity at 40˚C and gradually declines as temperature increases. In terms of temperature stability, Ku-19 shows higher temperature stability as compare to Ku-20. Slightly higher thermostable lipase as compare to Ku-19 and Ku-20 was obtained from extremely halophilic archaeon, *Natronococcus* sp. strain TC6 (Boutaiba *et al*., 2006). Lipase from *Natronococcus* sp was active maximally at 50˚C and retains more than 90% of original activity when incubated for 60 min at 50°C (Boutaiba *et al*., 2006).
- Inorganic salts are found to have direct effect on activity and stability of lipase. It may work as an inducer or inhibitor. Amongst all the inorganic salts tested, NaCl was found to have generally inducible action while other salts except NaF, EDTA, SDS led to decrease activity by 50% or more. Lipase inactivated in the presence of NaF, EDTA, SDS showed very less activity. The results are highly compatible with lipase from Marine *Vibrio fischeri* (Ranjitha *et al*., 2009).
- Mutagenesis is an important process for the improvement of organisms at industrial scale. Mutagenesis can be performed by physical and chemical agents. UV radiation is most widely used physical mutagen for strain improvement. Random mutagenesis UV showed that there was increase in lipase yield from Mk-

23 by about 3.4 U/ml. Increase in lipase yield by mutagenesis is much lower than what is shown in *Rhizopus oligosporus* IIB-63 in which mutantagenesis can able to increase lipase yield up to 32.42 U/ml as compare to wild type organism (Tehreema *et al*., 2011).

CHAPTER-6

Summary

The present work is focused on screening, isolation, biodiversity studies, study of growth kinetics, enzymatic potential studies, media optimization, partial purification, characterization and mutagenesis of halophilic microorganisms from Little *Rann* of Kutch with special emphasis on Lipase production. Following section indicates brief summary of results.

1. Total 30 isolates were obtained from saline water, mud and soil samples collected from little *Rann* of Kutch, near Surajbari Bridge and 24 isolates were obtained from excreta of wild ass, collected from "Indian wild ass sancturay", Dhrangadhra and Surendranagar.
2. All the isolates were studied in terms of colony, morphological and biochemical characterization. The organisms were diversified in terms of colony morphology and biochemical properties. Big sized colonies were round, irregular, flat, raised, slightly raised; sticky, white, pinkish etc. medium and small sized colonies have been also displaying variable colony characteristics. Isolates were Gram positive or Gram's variable.
3. Enzymatic profiling of all 54 isolates for enzymes like lipase, amylase, protease, cellulase and Chitinase was carried out. Among moderate halophiles, 9 isolates were producing lipase, 7 were producing amylase, 13 were producing protease, only 1 isolate produced cellulase and 2 chitinase. From extreme halophiles, 30 isolates produced lipase, 9 produced amylase, 10 produced protease while none of isolates among 30 produced cellulase or chitinase.

4. Potent amylase producer, Mk-21 produced maximum amylase after 72 hours of incubation in liquid media. Organism produced 0.7 U/ml amylase from crude sample. Media optimization data indicates that the organism produced maximum amylase at pH 6, temperature 30˚C, peptone as a nitrogen source and 1.2% starch. Km of enzyme was 1 mg/ml and Vmax 1.10 µm/min on the basis of substrate curve.

5. Efficient lipase producers, Mk-4, Mk-18, Mk-23, Ku-10, Ku-19 and Ku-20 were able to produce maximum lipase after 244 hours, i.e. during stationary phase. Mk-4, Mk-18 and Mk-23 produced maximum lipase at pH 5 to 6,

temperature 30-40˚C, NaCl 11%-13% (w/v) and 4-5% tributyrene, while Ku-10, Ku-19 and Ku-20 produced maximum lipase at pH 7-8, Temperature 30-40˚C, 10%-15% NaCl (w/v) and 4-5% Tributyrene (v/v).

6. Crude lipase was subjected to partial purification by Ammonium sulphate fractionation. Lipase from Mk-4 showed 1.33 fold purification and 33.3% yield, lipase from Mk-18 showed 3.44 fold purification and 70% yield, lipase from Mk-23 showed 1.98 fold purification and 42.2% yield. Lipase from extreme halophiles Ku-10 showed 2.91 fold purification and 55% yield, lipase from Ku-19 showed 5 fold purification and 54.15% yield and 4 fold purification and 50.2% yield was obtained from Ku-20.

7. Crude and partially purified lipase from Mk-4 and Mk-18 showed maximum activity at pH 5 and from Mk-23 at pH 5 and 6. Lipase from Ku-10 showed maximum activity at pH 6, Ku-19 at pH 7 and Ku-20 at pH 8.

8. Temperature optima data shows that Lipase from Mk-4, Mk-23, Ku-19 and Ku-20 were active maximally at 40˚ C temperature, lipase from Mk-18 was maximally active at 30˚C to 40˚C while lipase from Ku-10 was showing highest activity at 70˚C.

9. Temperature stability and urea denaturation point of view, lipase from Ku-10 was stable as compare to lipase from all other isolates.

10. Various salts except NaCl decreased enzyme activity and certain salts like NaF, EDTA and SDS showed direct adverse effect and reduced enzyme activity drastically.

11. Increase in lipase production up to 3.4 U/ml was achieved from Mk-23 after UV mutagenesis. UV mutagenesis was performed at 280 nm wavelengths, 90 second of exposure and by placing culture at the distance of 90cm from UV source.

CHAPTER-7

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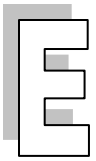

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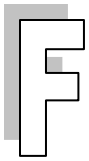

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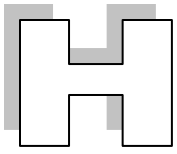

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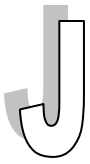

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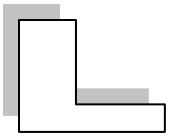

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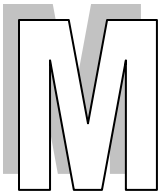

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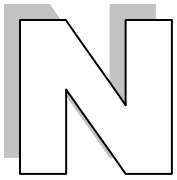

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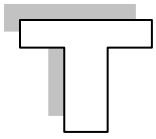

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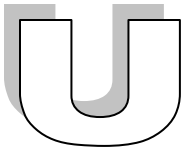

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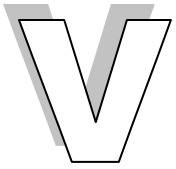

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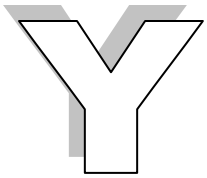

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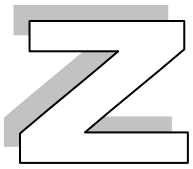

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